IN VIVO AND IN VITRO STUDIES ON BOVINE PAPILLOMA VIRUS

by

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A thesis submitted to the University of Glasgow in partial fulfilment of the requirements leading to the degree of Doctor of Philosophy.

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September, 1978.

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

(in order of first appearance in the text)

nm	nanometres
mm	millimetres
cm	centimetres
ml	millilitres
l	litres
m_r	apparent molecular weight
%	percent
HPV	human papilloma virus
BPV	bovine papilloma virus
SV40	simian vacuolating virus 40
EV	epididermo dysplasia verruciformis
RKV	rabbit kidney vacuolating virus
FPV	fringellae papilloma virus
ROPV	rabbit oral papilloma virus
SPV	Shope papilloma virus
OPV	ovine papilloma virus
TSTA	tumour-specific transplantation antigen
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
EM	electron microscope
PML	progressive multi-focal leukoencephalopathy
STMV	stumptailed macaque virus
UV	ultra-violet light
PV	papilloma virus
CPE	cytopathic effects
RDE	receptor destroying enzyme
PHFG	primary human foetal glial cells
^{o}C	degrees centigrade
CAM	chorioallantoic membrane
VP	viral protein
V-antigen	virion antigen
T-antigen	intranuclear tumour antigen
IF	immunofluorescent
CF	complement fixation
TSSA	tumour associated cell surface antigen
C-antigen	cytoplasmic antigen
TATA	cell surface specific graft rejection antigen

ID	immunodiffusion
SLE	systemic lupus erythematosis
DHR	delayed hypersensitivity reaction
CMI	cell mediated immunity
LT	lymphocyte transformation
LMIF	leukocyte migration inhibition factor
RBC	red blood cell - erythrocyte
WBC	white blood cell - leukocyte
HA	haemagglutination
HAI	haemagglutination inhibition
OMPV	owl monkey papovavirus
FEH	focal epithelial hyperplasia
mg	milligram
g	gram
HCl	hydrochloric acid
SDS	sodium dodec yl sulphate
V	volts
PTA	phosphotungstic acid
SD	standard deviation
SE	standard error
>	greater than
<	less than
CsCl	caesium chloride
TD	transforming dose
TID	transformation inhibitory dose
FBS	foetal bovine serum
BME	basal Eagle's medium
FBSC	foetal bovine skin cells
FBMC	foetal bovine meningeal cells
T	transformed
rpm	revolutions per minute
min	minute
hr	hour
$\log_{10}$	logarithm to base 10

NOTE Abbreviations specific to individual chapters appear in brackets following the first appearance of each expression and are not listed here.

ACKNOWLEDGEMENTS

I would like to thank:

: Professor W. F. H. JARRETT, my supervisor, for permission to use the facilities and materials needed for the research; for making time available to give advice, support and constructive criticism when circumstances permitted; and for the almost superhuman patience needed to read and improve this thesis and the scientific papers that result from it.

: Mrs E. LEIGHTON has provided highly specialised technical support in the performance of the transformation and transformation inhibition assays reported in Chapters 7 and 8.

: Mr. W. PENNY and staff who provided histopathological sections of tissues reported in Chapters 3, 4 and 5.

: Mrs. C. McLAY who provided thin sections as reported in Chapters 2, 10 and elsewhere in this thesis.

: Mr. A. FINNIE and staff who provided photographs and photomicrographs of tumours reported throughout the thesis.

: Mr. J. MORRISON and Mr. C. Wilson who provided the electron-micrographs in Chapters 2 and 9.

: Mr. J. MURPHY and staff for assistance in the conduct of bovine post-mortem examinations.

: Mr. D. McINNES for husbandry of the cattle reported in Chapters 3, 5 and 6.

: Mr. A. BRADLEY, Mrs. M. BOAG and staff for the husbandry of the experimental animals reported in Chapter 4.

: Professor D. LAWSON and staff who performed the intracerebral inoculations reported in Chapter 5.

: Dr. O. JARRETT and staff who provided expert advice on the cultural andvirological techniques reported in Chapters 7, 8 and 9.

: Mrs. M. McCREADIE, Mrs. M. TROY and Mrs. H. ZIKAKIS who together have typed this thesis.

: Dr. I. McCANDLISH who assisted with the lung histopathology.

: Mr. A. SPENCER who assisted with the kidney histopathology.

: Mrs. P. ROGERSON who assisted with the lymphoreticular histopathology.

: Mr. B. O'NEIL for assistance in ensuring the constant flow of the necessary materials reported throughout this thesis.

: Dr. D. ONIONS who assisted with immunological advice.

: Miss A. McRAE who performed haematological examinations.

: Mr. J. NEIL who performed the polyacrylamide gel electrophoresis techniques reported in Chapter 2, and has proved a valuable source of advice on virological aspects of the work.

: Mr. C.M. McCANCE of the Glasgow City Abattoir, for permission to carry out the survey reported in Chapter 2.

: Dr. H. LAIRD, who spent much valuable time teaching me electron microscopy, and subsequently giving advice on aspects of the ultra-structural work reported in this thesis.

: The Australian Meat Research Committee; the Commonwealth Government of Australia and the Agricultural Research Council of Great Britain who provided financial assistance which is gratefully acknowledged.

: Dr. M.A.S. JONES, executive officer of the Australian Meat Research Committee and Dr. R.W. GEE, director of the Australian Bureau of Animal Health who have given enormous encouragement and support.

I am indeed fortunate in having a patient and understanding wife, who, together with three children suffered considerably to allow this work to proceed to fruition. Their support was of critical importance and is acknowledged with gratitude.

H. R. C. MEISCHKE

September, 1978.

DECLARATION

I hereby declare that this thesis embodies the results of my own special work, that it has been composed by myself and that it does not include work forming part of a thesis presented successfully for a degree in Glasgow University or another University.

Signature .....

Date 21/9/78

SUMMARY

An abattoir survey reported an overall teat papillomatosis prevalence of 36 % in 721 cattle examined. Affected animals possessed an average of five papillomas on each of two affected teats. Significant sex differences occurred in the prevalence of the disease, and nulliparous female cattle had significantly fewer affected teats than parous females. Morphologically separable lesions were seen and on histological examination were shown to be papillomas, fibropapillomas and focal epithelial hyperplasia-like lesions. Bovine papilloma virus was extracted from all three lesion types. Electron microscopic examination revealed that BPV extracted from teat papilloma, fibropapilloma and focal epithial hyperplastic lesion had significantly different particle diameters. Polyacrylamide gel electrophoresis of BPV from cutaneous fibropapillomas showed a major capsid protein band which has a consistently higher apparent molecular weight than teat lesion extracts. Epidemiological evidence suggested the presence of at least two separate virus/host interactions were present since the distributions of the individual teat lesions differed in both number and shape of curve.

When single case, single lesion type BPV extracts were inoculated into experimental calves, four morphologically separable lesions resulted. BPV from teat and cutaneous fibropapillomas produced cutaneous fibropapillomas which were morphologically indistinguishable from each other; BPV from anogenital fibropapillomas produced cutaneous fibropapillomas morphologically separable from those above; BPV from teat papilloma and focal epithelial hyperplastic lesions produced papilloma and focal epithelial hyperplastic lesions respectively on transmission to normal bovine skin. BPV was extracted from all transmitted lesions.

Experimental calves were challenged with BPV from all five lesion types and a differential immunity was demonstrated. The fibropapilloma calves were immune to challenge with most but not all fibropapilloma isolates, but susceptible to challenge with teat papilloma and focal epithelial hyperplasia isolates. Conversely papilloma and focal epithelial hyperplasia BPV infected calves remained susceptible to all fibropapilloma isolates and regression of fibropapillomas was not accompanied by the regression of the former lesions. It was considered that two broad virus categories occurred with papilloma and focal epithelial hyperplasia BPV forming one and fibropapilloma BPV the other category. Within these two categories there were morphological and developmental differences suggesting further heterogeneity which may be confirmed with the use of larger numbers of experimental animals.

When inoculated intracerebrally, two fibropapilloma BPV isolates produced meningiomas. One isolate was from a single case of cutaneous fibropapillomas. The other was from cutaneous fibropapillomas which appeared along scarification lines of a calf experimentally inoculated with BPV extracted from a pooled sample of alimentary lesions. A third calf, intracerebrally inoculated with BPV from single case teat focal epithelial hyperplastic lesions, developed similar lesions at intradermal sites but did not develop a meningioma.

In vitro, foetal bovine skin, conjunctiva, palate and meninges derived cell cultures were shown to be sensitive to transformation by BPV extracted from fibropapillomas but not papillomas and focal epithelial hyperplastic lesions. Only two skin cultures were sensitive and both of these came from near-term foetuses. Cultures from the pia mater and arachnoidea were sensitive while dura mater derived cultures were not. The in vitro transformation assay was developed and shown to be more sensitive and precise than haemagglutination, however the two systems probably measure different parameters. No significant differences occurred in the transformation titre of two BPV isolates when different tissues of the same foetus and the same tissues of different foetuses were used.

BPV-transformed cells were shown to be tumourigenic in nude mice but not in calves following intracerebral, subcutaneous, intradermal and submucosal injection. However, inoculated calves showed an increased in vivo resistance to challenge with fibropapilloma derived BPV but not papilloma or focal epithelial hyperplasia derived BPV. BPV-transformed cells showed an increased lifespan when compared with control cultures. The morphological changes induced by BPV were heritable in all cultures and, with one exception, persisted throughout the in vitro lifespan of each culture. One foetal skin culture showed a reversion to normal morphology approximately six months following infection by BPV. The revertant cells were resistant to re-transformation by BPV even though paired control cultures remained transformation-sensitive.

In vitro transformation by BPV could be inhibited by sera from fibropapilloma bearing calves. Transformation-inhibition (TI) was shown to be complement dependent but virus independent over a wide range of dilutions. TI titres first appeared in sera from calves with fibropapillomas concurrently with the first appearance in biopsy samples of cell mediated regression as evidenced by lymphocyte accumulation in the fibroma component. TI titres persisted for 4 - 8 weeks following complete regression of fibropapillomas. In BPV - induced

meningiomas, no histological evidence of rejection could be seen. TI titres could not be detected in serum samples following complete rejection of cutaneous fibropapillomas on the same calves despite the presence of progressively developing meningiomas. Meningiomas appeared to originate in the pia mater since in both calves, the dura mater was uninvolved. TI antibody activity could be adsorbed from high titre serum using fibroma, fibropapilloma and meningioma tissue suspensions as well as in vitro transformed cells. TI antibody activity was not reduced following absorption with papilloma, focal epithelial hyperplastic, normal bovine and foetal bovine tissue suspensions, foetal bovine cell cultures, as well as all clarified and purified BPV suspensions, with one exception. The cutaneous fibropapilloma derived purified virus suspension contained sufficient BPV to over-ride the normal virus concentration independence of the assay system.

BPV was shown to be resistant to pasteurisation in vivo using separate extracts of all the major lesion types. In vitro, fibropapilloma derived BPV showed a ten-fold fall in transformation titre following 4 hours at $60^{o}C$ and all transformation activity ceased after 30 minutes at $80^{o}C$. BPV was detected in two of six milk samples purchased at retail outlets. The likely widespread exposure of humans to BPV in milk, coupled with a similarity between some teat lesions and oral human lesions led to the suggestion that the question of whether BPV can infect man be investigated further.

Only 3 of 44 cultures tested for in vitro BPV replication showed BPV in the medium. This occurred between 24 - 28 days post infection. Radio-labelling with tritiated thymidine showed the presence of a peak in BPV containing medium purified in CsC1 . The results of radio-labelling, buoyant density and electron microscopy suggested that BPV had been cultivated in vitro. The results were far from conclusive, but the combination of prolonged contact inhibition and severe stress may have contributed to the phenomena observed.

Chapter 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

"When viruses interact with cells they bring about alterations in the DNA content of the cells. Though the precise mechanisms may be different this is also true of chemical carcinogens and radiation. The end result is determined partly by the nature of the damage but also in part by the capacity of the cell to repair the damage. These events may play an important part in the initiation of neoplasia." (Harnden, 1976).

1.1 The Papovaviridae

The term papovavirus was first used by Melnick (1962) to denote a group of viruses with common characteristics. Papilloma, polyoma and vacuolating agent (SV40) each provide their first two letters to form the group name, Papova. The International Commission for the Nomenclature of Viruses officially approved the name Papovaviridae which is now used to denote a family of viruses that is subdivided into two genera, papillomavirus and polyomavirus, with rabbit (Shope) papillomavirus and polyomavirus the respective type species.

1.1.1 General Properties of Papovaviruses

The viruses are 40-57 nm in diameter and, as determined by negative staining, the outer shell has a symmetry of the T = 7 icosahedral surface lattice and is composed of 72 morphological subunits (Finch and Klig 1965; Anderer et al., 1967). The viruses are ether stable (containno essential lipids). They contain DNA, present as a covalently closed duplex molecule, within the core of the virus particle. Papillomaviruses have DNA of a molecular weight close to 5×10^6 daltons, higher than the molecular weights of SV40 (3.6×10^6) (Tai et al., 1972) and polyoma (3.0×10^6) (Weil and Vinograd, 1963). The DNA's of the papovaviruses are infectious and are able to transform cells in vitro (McCutchan and Pagano, 1968; Crawford et al., 1964; Bourgaux et al., 1969; Aaronson and Todaro, 1969; Aaronson and Martin, 1970).

1.1.2 Original Isolation of the Papovaviruses

Table 1 lists the known members of the papovaviridae and their source of original isolation. At the point of writing, the number of separately

identified papovaviruses stands at twenty-seven. Evidence for existence of several substrains within recognised types is mounting as well as the relatedness of separately recognised types.

Three recently isolated strains of JCV, MAD 1, MAD 2 and MAD 4 have shown a differential neurooncogenicity following intracerebral inoculation in newborn hamsters. MAD 1 and MAD 2 produced medulloblastomas of the cerebellum while MAD 4 tended to produce pineal gland tumours (Padgett et al., 1977). Within the papillomavirus group, the existence of separate viruses causing lesions restricted to certain anatomical areas and tissues has been known for a long time. The rabbit has both a skin papillomavirus and an oral papilloma virus as does the dog. More recently, the existence of several different human papilloma viruses (HPV) has been reported. Virus from genital and laryngeal papillomas, and virus from epidermodysplasia verruciformis (EV) have been shown to be different from virus extracted from the common skin wart. (Almeida et al., 1969; ZurHausen et al., 1974; Orth et al., 1978; Gissman et al., 1977; Orth et al., 1977). This thesis will contain evidence for the existence of several types of bovine papilloma virus (BPV) (Meischke, 1978a-e).

Several types of polyomaviruses have been compared immunologically, biochemically, in vivo and in vitro. The results show varying degrees of relatedness between members of the group. Cross reactions among the V antigens of SV40, BKV, JCV and RFV have been observed by haemagglutination inhibition (HI) (Gardner et al., 1971; Walker et al., 1974), neutralisation (Bernhardt et al., 1975; Dougherty and di Stefano, 1974), fluorescent staining (Takemoto and Mullarkey, 1973) and immune agglutination (Albert and Zu r hein, 1974; Field et al., 1974; Gardner et al., 1971; Penny and Narayan, 1973). Cross reactions among the non-V antigens have also been observed. The intranuclear T antigen has been shown to be virus related and not host cell related. The nuclear T antigens of SV40, RFV, BKV and JCV have been shown to be similar with strong cross reactions observed by fluorescent staining, while polyoma T antigen is different from all four (Shah et al., 1973; Takemoto and Mullarkey, 1973; Walker et al., 1973). The virus induced tumour cell antigen, U, is also crossreactive with anti SV40 U antiserum reacting with BKV and JCV infected cells (Takemoto, 1975 personal communication, cited by Padgett and Walker, 1976). The virus specific

TABLE 1. THE SOURCE OF ORIGINAL ISOLATES OF PAPOVAVIRUSES

VIRUS	SOURCE OF ORIGINAL ISOLATION	REFERENCE
Polyoma	parotid gland tumours of mouse	Gross (1953), Stewart (1953).
SV40	monkey cell cultures	Sweet and Hilleman (1960).
BKV	urine of renal transplant patient monkey cell cultures, human foetal kidney, lung, brain cultures	review by Padgett and Walker (1976).
JCV	human brain with progressive multifocal leukoencephalopathy	Padgett et al., (1971).
RFV	urine of renal transplant patient	Dougherty (1976).
MMV	reticulum cell sarcoma of brain and urine of patient with Wiskott-Aldrich syndrome	Takemoto et al., (1974).
STMV	stumptailed monkey kidney cultures	Rangan et al., (1974).
HD	- monkey kidney cell cultures	Wal deck & Sauer (1977).
K virus RKV	respiratory tract and faeces of mice rabbit kidney cell cultures	review by Andrewes and Pereira (1972)
unnamed papova viruses	swine papovavirus	Newman and Smith (1972)
	lymphocytes of measles patients	Lecatsas et al., (1976)
	brain of patient with Creutzfeldt-Jakob disease	deReuck et al., (1976)
	bladder epithelium of patient with acute haemorrhagic cystitis of childhood (AHC)	Hashida et al. (1976).
	kidney cell cultures of the owl monkey	Daniel et al., (1976).

TABLE 1. (Cont'd). THE SOURCE OF ORIGINAL ISOLATES OF PAPOVAVIRUSES

VIRUS	ANIMAL	TUMOUR	REFERENCE
FPV	chaffinch	skin papillomas	Osterhaus et al., 1977.
	opossum	skin papillomas	Koller, 1972.
Shope	rabbit	skin papillomas and carcinomas	Shope, 1933.
ROPV	rabbit	oral papillomas	Parsons and Kidd, 1943.
	dog	skin papillomas	Watrach, 1969.
	dog	oral papillomas	Chambers and Evans, 1959.
	chimpanzee	focal epithelial hyperplasia	Orth, 1976.
OPV	sheep	skin fibropapillomas	Head, 1965.
	goat	skin papillomas	Davis and Kanper, 1936.
	goat	udder papillomas and carcinomas	Moulton, 1954
	deer	skin fibromas;fibro-papillomas	Shope et al., 1958.
Graffi	hamster	skin papillomas	Graffi et al., 1970.
	pig	genital papilloma	Parish, 1961.
	monkeys	skin papillomas	See section 1, 3, 17.
BPV	cattle	skin fibropapillomas	Creech, 1929.
	cattle	anogenital fibro - papillomas	McEntee, 1952.
	cattle	skin papillomas	Barthold et al., 1974.
	cattle	alimentary papillomas	Jarrett et al., 1978.
	cattle	teat papillomas, fibro-papillomas and rice-grain lesions	Meischke, 1978.
HPV	human	skin papillomas	Cuiffo, 1907.
		focal epithelial hyperplasia	Praetorius-Clausen, 1972.
		anogenital and oro-laryngeal papillomas	Rowson and Mahy, 1967.

tumour-specific transplantation antigen (TSTA) demonstrated by protective experiments in hamsters, however, appears not to cross react between JCV, BKV and SV40 induced tumour cells (Padgett et al., 1977; Padgett and Walker, 1976). While virus proteins of SV40, BKV and JCV are antigenically related, the major protein of BKV is smaller than that of SV40 but the minor proteins have similar molecular weights (Mullarkey et al., 1974; Walker et al., 1974). Using iodination or tryptic digestion, some of the polypeptides of SV40 and BKV showed differences in spite of similar molecular weights (Weiss et al., 1975). An 11 - 12% homology in the "late" region of SV40 DNA was found between the DNA of SV40 and BKV (Khoury et al., 1975).

From the above examples, it can be seen both that there are inter-relationships between different members of the papovaviridae and marked heterogeneity between different isolates of the same papovavirus. Former clearly defined differences, still listed in current textbooks, between the polyoma-viruses and the papillomaviruses are now less distinct with considerable overlap between the two groups in most parameters. Polyoma and SV40 are two of the most intensively studied viruses in history, for example, the complete nucleotide sequence of SV40 DNA has been published (Fiers et al., 1978). There are in excess of 1000 references concerning the polyoma group of viruses which have been published since 1973. This thesis will concentrate on comparative aspects of the papovaviruses which bear directly on properties displayed by bovine papillomavirus (BPV).

1.2 Polyomaviruses in Vivo - host range and oncogenicity.

The polyomavirus group of the papovaviridae display a wide range of oncogenicity in vivo. Some members are nononcogenic while others are oncogenic in a number of animal species. In general, those oncogenic members produce adenomas, carcinomas and fibrosarcomas or a mixture of these tumours in their natural host and other species. The type of tumours produced varies with the route of inoculation, the relative oncogenicity of the virus and the susceptibility of the animal. However, with rare exceptions, the viruses produce tumours restricted to epithelial and mesenchymal tissues and their equivalents within the central nervous system. Although there are a great many interrelationships between individual members of the group, the following pages will attempt to conform to the most recently reported

sub-groupings of the polyomaviruses.

1.2.1 Polyomavirus

Although neonates and certain strains of mice are highly susceptible to polyoma induced neoplasia (Gross, 1970; Rowe et al., 1962), generally the virus is latent with tumour induction in outbred and inbred strains a very rare occurrence (Gross, 1970). The wide range of tumours induced by polyoma virus in the mouse includes:

sarcomas	-	salivary glands; mucous and serous glands of the head and neck; kidney; subcutaneous tissue; bone and cartilage.
carcinomas	-	salivary glands, mucous and serous glands of the head and neck; hair follicle; adrenal medulla.
adenomas	-	salivary glands; mucous and serous glands of the head and neck.
also	-	adenocarcinomas of mammary gland, mesotheliomas, endotheliomas, epithelial thymomas, haemangioendotheliomas.

However, the most common disease induced by polyoma in the mouse is bilateral, multilobulated neoplasms of the parotid gland. (Gross, 1970; Stewart, 1960).

Polyomavirus is also oncogenic in other species. Table 2 lists the species and the types of tumours produced by polyomavirus.

Polyoma produces primarily carcinomas and sarcomas of a wide range of tissues and organs in its natural host while the mesenchymal tumours, sarcoma and fibroma are produced following experimental transmission to other species.

TABLE 2: ONCOGENICITY OF POLYOMAVIRUS IN NON-MURINE SPECIES

Species	Tumour induced	References
hamsters	sarcoma	Eddy et al., (1958) Berman (1967)
newborn hamster	meningeal sarcoma	Rabson and Kirschstein (1960)
rat	sarcoma	Eddy et al., (1959)a
mastomys	sarcoma	Rabson et al., (1960)
ferret	sarcoma	Harris et al., (1961)
guinea pig	sarcoma	Eddy et al., (1960)
rabbit	fibroma	Eddy et al., (1959)

1.2.2 SV40

As with polyoma, SV40 produces a latent infection in Rhesus, Cynomol gus and Ceropithecus monkeys (Eddy et al., 1962; Sweet and Hilleman, 1960). However, when given to suckling grivets, baboons or Rhesus monkeys, SV40 produces fibrosarcomata. (Andrewes and Pereira, 1972).

SV40 is oncogenic in other species. Table 3 lists the species and types of tumours produced by SV40.

TABLE 3: ONCOGENICITY OF SV40 IN NON-SIMIAN SPECIES

Species	Tumour induced	References
hamster newborn	choroid plexus papilloma, ependymoma, sarcoma, fibrosarcoma	Kirschstein and Gerber, (1962) Unterharnscheidt et al., (1964) Berman, (1967); Eddy, (1962); Duffel et al., (1964).
weanling	lymphocytic leukemia, lymphosarcoma, reticulum cell sarcoma, osteogenic sarcoma, anaplastic sarcomas of various organs	Diamandopoulos, (1973).
Mastomys	ependymoma	Rabson et al., (1962).

SV 40 has been accidentally inoculated into large numbers of people as a result of contamination of tissue culture derived poliomyelitis vaccine. A full discussion and review on this subject has been written by Shah and Nathanson (1976). The virus has been isolated from the tissues and fluids of patients with a variety of tumours. Two cases of PML have yielded viruses which are antigenically indistinguishable from SV40 but display minor DNA differences confined to the hypervariable regions of SV40 DNA (review by Weiner and Narayan, 1974). SV40 was isolated from three metastatic melanomas of a patient with no history of vaccination likely to contain SV40. Pleural exudate contained SV40 anti-V and anti T antibodies (Soriano et al. 1974). SV40 related antigens, T and V were found in three of seven human meningiomas when examining the nuclei of cultured cells derived from them. Fusion of these cells with SV40 permissive cells led to the appearance of SV40 V antigen in a low percentage of cells (Weiss et al., 1975). It has already been stated that the immunological relatedness of SV40, BKV, JCV and RFV, and the existence of several sub-types within each of these, renders the task of distinguishing these viruses in isolations of human tumours reported prior to 1973 difficult or impossible.

1.2.3 BKV, RFV and MMV

Since the viruses RFV and MMV are closely related to BKV, they will be discussed together with BKV in this section - MMV, RFV and BKV will collectively be called BKV (Howley et al., 1975; Miao and Dougherty, 1977a) and b)).

A substantial number of patients subjected to long term immuno-suppression show evidence of active BKV infection. Gardner et al., (1971) made the original isolation of BKV from the urine of such a patient. That immunologically crippled patients, either due to therapy (kidney transplant patients) or due to disease syndromes (e.g. Wiscott-Aldrich syndrome) show rising antibody titres to BKV and urinary excretion of the virus may be a result of primary infection or delayed latent infection (review by Padgett and Walker, 1976). Antibodies to BKV are present in 83% of the human population of England (Gardner, 1973) and a similar frequency has been found in the United States (Mullarkey and Takemato, 1973). The presence of BKV in the

human population is widespread but the question of whether BKV produces disease in man has not yet been fully answered. BKV DNA sequences were detected in five of twelve human tumours and three of four human tumour cell lines (Fiori and di Mayorca, 1976). In one kidney transplant patient, the donor ureter showed fibrosis and an ulcerated epithelium, histologically showing large intranuclear inclusions which contained papovavirus particles when examined under the EM. (Coleman et al., 1973; Garner et al., 1971). Similar observations have been made by ZuRhein and Varakis (1974). A rise in antibody titre to BKV has been demonstrated in patients with Guillain-Barré syndrome (acute inflammatory polyradiculoneuropathy) in the absence of seroconversion against other candidate viruses (Noordaa and Wertheim-van Dillen, 1977).

Table 4 list the oncogenicity of BKV viruses in animals other than man.

TABLE 4: ONCOGENICITY OF BKV VIRUSES IN NON-HUMAN SPECIES

BKV	Species	Tumour induced	References
BKV	newborn hamster	sarcoma	Shah et al., 1975
	newborn hamster	no tumour	Takemoto et al., 1974
BKV(MMV)	newborn hamster	no tumour	
BKV	newborn hamster	fibrosarcoma	Noordaa 1976
BKV(MMV)	newborn hamster	ependymoma	Costa et al., 1976
BKV(RFV)	newborn hamster	sarcoma	Dougherty 1976
BKV	newborn hamster	ependymoma and insulinoma	Uchida et al., 1976

The above summary indicates that the BKV, MMV and RFV group of papovaviruses have a reduced pathogenicity and oncogenicity in the natural host and experimental animals when compared with SV40 virus. There may be some differences between BKV, MMV and RFV in their oncogenicity in hamsters.

1.2.4 JC Virus

Unlike the BKV, MMV, RFV group of viruses above, there is strong evidence that JCV does cause disease in man, its' natural host. JCV is strongly associated with the rare, demyelinating disease, progressive multifocal leukoencephalopathy (PML). JCV particles have been repeatedly demonstrated in the nuclei of oligodendrocytes in and around the lesion both by E M and immunofluorescent staining (for review, see Padgett and Walker, 1976). Late PML lesions frequently contain giant astrocytes morphologically resembling the malignant astrocytes of pleomorphic glioblastomas (Astrom et al., 1958; Zurhein, 1969). PML is a disease commonly associated with patients showing an impaired immune system either due to chronic disease or immunosuppressive therapy following kidney transplantation (Zurhein and Varakis, 1974a; Legrain et al., 1974; Manz et al., 1971; Padgett and Walker, 1976). JCV has been isolated in a case of PML which also showed multiple gliomas, similar to the lesions produced in the hamster following intracerebral inoculation of JCV (Castaigne et al., 1974; Walker et al., 1973). Seroepidemiologic evidence indicates that BKV and JCV circulate completely independently, supporting the view that they are quite distinct viruses (Brown et al., 1975).

JCV is oncogenic in animals other than man and Table 5 summarises the information available to date.

TABLE 5: ONCOGENICITY OF JCV IN NON-HUMAN SPECIES

JCV Strain	Species	Tumour induced	References
JCV(MAD1)	newborn hamster	malignant glioma* rarely ependymoma	Walker et al., 1973
JCV(MAD2)	newborn hamster	as above	
JCV(MAD4)	newborn hamster	50% pineocytoma. 50% malignant glioma, rarely ependymoma, and extracranial neuroblastoma, unclassified tumours of the ileum	Padgett et al., 1977
JCV	newborn hamster	sarcomas, mainly internal but some subcutaneous	Padgett and Walker, 1976.

* include medulloblastoma, glioblastoma, unclassified primitive tumours.

While BKV and JCV viruses are recent discoveries, it is already clear that each has several variants or strains, each showing differing oncogenicity in vivo. It is probable that the information presented here does not represent the complete host range or oncogenicity of either virus but that future work will expand present knowledge.

1.2.5 Other Viruses of the Polyoma Group

(i) STMV

The stumptailed macaque virus (STMV) is a recently isolated papovavirus always present in the kidneys of its natural host, both in post-natal and in foetal life (Shah et al., 1977). It is present in uninoculated kidney cell cultures of the stumptailed macaque and has been shown to be distantly related to SV40 and BKV. It is unique among the papovaviruses in that many virions appear to possess an additional outer envelope (Reissig et al., 1976). No information is yet available in the literature as to its in vivo effects.

(ii) Owl Monkey Papovavirus

One report (Daniel et al., 1976) describes the isolation of this virus, that it is not pathogenic for rabbits and that a 100% mortality results within seven days following inoculation of newborn hamsters but without specific histopathologic lesions.

(iii) HD virus

HD virus was isolated from a permanent monkey kidney cell line derived from Cercopithecus aethiops monkey (VERO-cells). It has been shown to have a smaller diameter than SV40 under the EM, and its' DNA has a molecular weight of 3.1×10^6 daltons (compared to 3.6×10^6 for SV40 DNA) (Waldeck and Sauer, 1977). No information is yet available as to its in vivo effects.

(iv) Non Oncogenic Polyomaviruses

Excluding the STMV, HDV and the owl monkey papovavirus because of their recent discovery, a number of the polyoma group of viruses show no oncogenicity.

K-virus is commonly found in mice and in newborn mice; it produces pneumonitis and hepatic cell changes but not in adult mice (Fisher and Kilham, 1953; Kilham and Murph, 1953).

Porcine Papovavirus has not been shown to be oncogenic (Newman and Smith, 1972).

Latent Rat Virus, or the Toolan H-1, H3 etc. virus is now thought to be a parvovirus but remains a possible papovavirus since 66% of its DNA is double stranded. The virus has been found in a number of human tumours and is osteolytic, so called because it causes Mongolism in neonatally inoculated hamsters (Toolan, 1960).

RKV Virus

The rabbit kidney vacuolating virus has been found in normal tissues as well as Shope papillomas. It is not known to be pathogenic in any species (Hartley and Rowe, 1964; Ito et al., 1968).

1.3 Papillomaviruses in vivo - host range and oncogenicity

The papillomavirus group of the papovaviridae display a wide range of oncogenicity in vivo. Some members produce benign epithelial hyperplastic lesions restricted to their host species while others are oncogenic in a number of animal species. In general, those oncogenic members produce tumours restricted to epithelial and mesenchymal tissues and their equivalents in the central nervous system. It is now accepted that any one species may have a number of different and often partly related papillomaviruses causing various lesions. The following review will conform to the accepted practice of naming the viruses according to their species of original isolation.

1.3.1 Human Papilloma Virus (HPV)

Previously, all papillomas of man were thought to be due to one virus (Andrewes and Pereira, 1972). Recent literature now indicates that there may be several viruses involved, some causing morphologically separable lesions. Some lesions proceed to carcinomas and other malignancies while others remain benign. HPV has been shown to produce papillomas in the dog (see Section 1.3.4(c) but insufficient work has been done to fully determine

the experimental host range of HPV. The only other reports date from the 1930's and show HPV to be non-transmissible to dogs, guinea pigs, monkeys and calves (Biberstein, 1930).

1.3.2 Verruca vulgaris, plana, plantaris

The common cutaneous or skin papilloma can occur on different anatomical sites, giving rise to differences in macroscopic and microscopic appearance and corresponding pathological terms (Review by Rowson and Mahy, 1967). Papillomas are common among school children and young adults. Table 6 illustrates the epidemiological nature of verrucae in Scotland from 1970 to 1976 (source Anon. 1977).

TABLE 6: PREVALENCE % OF VERRUCA IN SCOTTISH SCHOOL ENTRANTS AND SCHOOL LEAVERS FROM 1970 TO 1976.

YEAR	SCHOOL ENTRANTS				SCHOOL LEAVERS			
	Boys		Girls		Boys		Girls	
	P*	N+	P	N	P	N	P	N
1970	2.39	44.9	2.44	42.8	5.67	32.3	6.24	32.4
1972	2.58	40.9	2.37	39.2	5.87	33.8	6.54	33.7
1973	2.36	41.8	2.32	39.7	6.10	32.1	6.37	32.1
1974	2.45	40.9	2.52	39.5	5.66	34.6	5.91	34.5
1975	2.12	37.7	2.43	35.5	9.89	19.2	8.69	18.9
1976	2.20	37.1	2.27	35.0	8.83	19.0	7.89	18.9

* P = prevalence (%)

\+ N = number examined ($x10^3$)

Source - Anon. 1977.

Table 6 shows a prevalence of between 2.12 and 2.58% of verrucae in children of age 5 - 6 years on school entry and between 5.67 and 9.89% of verrucae in children leaving school at 14 - 15 years of age. There is a consistency in these figures that suggests a stable epidemiological situation with no

significant sex differences. The prevalence of verrucain the older age groups suggests that most, if not all school leavers will have been exposed to HPV by the time of puberty. The figures are in general agreement with the prevalence of 7 - 10% reported in school children by Rowson and Mahy (1967).

Widespread controversy still exists over the precise mechanism of rejection of verruca and hence the value of various immunological parameters in estimating previous human exposure to HPV. Different HPV antigen preparations have led to widely differing estimates of antiviral antibody in patients with verruca. Anti HPV antibody was detected in 10 - 50% of patients with verruca by Ogilvie (1976) and 20 - 100% of patients with regressing verruca by Pyr honen (1976). Pyr: honen (1976) concluded that nearly everybody had been infected with HPV by the age of 20.

In a comparison of the normal skin surface of paired wart affected and control patients, Gloor et al., (1976) found certain significant differences (1) Wart patients had a significant reduction in the percentage amount of squalene in the skin surface lipids (2) Wart patients had a reduced alkali neutralisation time (3) The degree of water moistening of the skin was less in wart patients.

In one third of patients, verruca may persist for 7-9 years (Cubie and Bunney, 1976) while the balance regress within two years (Rulison, 1942; Massing and Epstein, 1963). However verruca may occur following wart regression in up to one half of patients. Papillomas have occasionally been reported to cause erosion of surrounding tissues, even bone (Shah et al.,1976), however, malignant transformation of skin papillomas has never been reported (Rowson and Mahy, 1967). The incidence of verruca is increased in patients with decreased cellular immunity as for example, those who have had renal transplants or lymphomas (Perry and Harman, 1974; Spencer and Andersen, 1970; Reid et al., 1976).

1.3.4 Condylomata acuminata

Condyloma acuminatum, the anogenital wart of man, may affect both epithelial and mucosal surfaces and is commonly venereally transmitted (Oriel, 1971; Lever and Shaum burg-Lever, 1975). Condylomata are stimulated to grow and involve adjacent surfaces during pregnancy and regress

following delivery. Prevalence at delivery has been estimated at 1 - 2% (Cook et al., 1973). On occasions condyloma may become extensive giving rise to the rare giant condyloma of Buschke and Lowenstein (1932). Malignant transformation of condyloma has been reported regularly in the literature. Anogenital warts are associated with up to 5% of vulval carcinomas and 15% of penile carcinomas (Underwood and Hester, 1971; Rhatigan et al., 1972). Occasional reports also show conversion to malignancy of cervical and anorectal condyloma (Kazal and Long, 1958; Sawyers, 1972; Fitzgerald and Hamit, 1974). Rarely, condyloma can involve the urethra (Gartman, 1956; Lindner and Pasqier, 1954; Dretler and Klein, 1975) and the bladder (Pettersson et al., 1976; Kleiman and Lancaster, 1962; Lewis et al., 1962).

Anogenital warts have shown an increasing prevalence in recent years, and Ghosh (1977) cites a prevalence of 5.34% in patients presenting at sexually transmitted disease clinics in 1976.

1.3.4 Alimentary and Respiratory Tract Papillomatosis

Papillomas of the upper alimentary and respiratory tract form a complex of clinical and pathological syndromes often referred to using widely varying names. They are grouped here for review purposes.

a) Focal Epithelial Hyperplasia

Focal epithelial hyperplasia (FEH) has been reported in the oral cavity in man and chimpanzee (see section 1.1.2) and a similar lesion occurs on the bovine teat (Meischke, 1978 and elsewhere in this thesis). Histologically, these lesions are not papillomas but discrete papules of hyperplastic epithelium which may coalesce to cover large areas (Praetorius-Clausen, 1972). Papilloma virus has been isolated from all three animals in which the lesion has been reported (section 1.1.2; Hanks et al., 1972; Kuffer and Perol, 1976; Praetorius-Clausen and Willis, 1971 and elsewhere in this thesis).

The prevalence of human FEH varies widely in different parts of the world. South American Indian children showed a prevalence of 33.8% (Soneira and Fonseca, 1964) and a restricted population of Greenlandic Eskimos of all ages, 35.8% (Praetorius-Clausen, 1970). A far lower prevalence has been reported in Caucasians. Axell (1976) found 0.11% overall prevalence in a large survey of Swedes with the highest prevalence of 0.40% in

people older than 75 years of age. In his review, Praetorius-Clausen (1973) concludes that the aetiology of FEH is viral with a probability that a genetic factor affecting susceptibility to FEH is also involved.

b) Alimentary Tract Papillomatosis

Reports of oral condyloma acuminatum are rare and are generally associated with infection from anogenital condylomas (Doyle et al., 1968; Knapp and Vohara, 1967; Seibert et al., 1969). Oral manifestations of verruca plana are limited to epidermodysplasia verruci formis which will be discussed later.

Oral verruca vulgaris is a rare lesion among the general population with a prevalence of 0.10% reported by Axell (1976). However it shows an increased prevalence in old people - a 6.2% prevalence was reported by Bhaskar (1968). Multiple oral papillomatosis of possible genetic aetiology is seen in "nevus unius lateris" and the "focal dermal hyperplasia syndrome" (Brown and Gorlin, 1960; Waldon, 1970). Multiple papillomatosis of other parts of the alimentary tract has also been reported, for example the palate (Lambert et al., 1976) and the oesophagus (Darani and Villa, 1976). Only one report describes the transformation to malignancy of oral papillomas (Samitz et al., 1967).

c) Respiratory Tract Papillomato sis

Papillomatosis of the larynx is the commonest tumour of the larynx in children as well as a significant disease in adults (Bone et al., 1976). It is a particular problem because of 1) the obstructive location 2) the multiplicity 3) the high recurrence rate of lesions and 4) the spread of lesions to the trachea and bronchi (Al - Saleem et al., 1968).

Several possible modes of infection have been published. Reports show some associations (1) 42-62% of patients with laryngeal papillomatosis had concurrent or prior skin warts (Kaufman and Baloch, 1969; Hajek, 1956) (2) Some authors have suggested an association between canine papillomatosis and human laryngeal papillomas (Uhlman, 1923; Ischikawa, 1936; Resler and Snow, 1967). (In a survey of 100 dogs, Pyrhönen (1976) found serological evidence of HPV infection in almost 25%.) (3) Laryngeal papillomatosis in children has often been reported in association with mothers showing condyloma

during pregnancy and partuition (Wallenborn, 1976; Cook et al., 1973). Further evidence for the association of laryngeal and anogenital papillomatosis comes from comparison of virus DNA (Zur Hausen et al., 1974; Delap et al., 1976; Gissmann et al., 1977), viral proteins (Pfister et al., 1977), and immunological techniques (Almeida et al., 1969).

Malignant transformation of laryngeal papillomas has been reported following radiation therapy (Fechner et al., 1974) but also in its' absence (Shapiro et al., 1976; Zehnder and Lyons, 1975).

1.3.5 Epidermodysplasia Verruci formis (EV)

While multiple papillomatosis of possible genetic aetiology has been mentioned earlier with respect to two syndromes (see section 1.3.4 (b)), EV is sufficiently well studied to be treated separately. EV is a dermatosis where verruca plana papules appear usually by puberty and disseminate to a great extent in early adult life (Jablonska and Milewsky, 1957). HPV has been identified in such lesions by electron microscopic and virological methods (Yabe and Sadakane, 1975). The viral infection has been incriminated in the early development of skin malignancies which appear in 25% of cases even though HPV or HPV DNA has not been detected in malignant cells (Yabe and Sadakane, 1975; Jablonska et al., 1972 and 1976).

In another genetic disease, xeroderma pigmentosum, patients show skin cancers at an early age on light exposed areas due to defective repair of UV damaged DNA (Cleaver, 1968; Robbins et al., 1974). In EV patients, skin cancers also appear first on sun exposed areas and such patients also show defective repair of UV damaged DNA (Hammar et al., 1976). HPV extracted from EV patients has been shown to be different from HPV extracted from verrucavulgaris lesions (Orth et al., 1977). The available evidence supports the theory that EV is a multifactorial disease in which HPV and defective DNA repair play important roles in generating malignancies. Parallel models exist in rabbit Shope papillomatosis and bovine alimentary papillomatosis to be discussed later.

1.3.6 Shope Papilloma Virus

Shope papillomas are commonly encountered in wild cottontail rabbits (Sylvilagus spp.), and experimentally, the virus produces papillomas in hares (Lepus spp.) and domestic rabbits (Oryctolagus cuniculus)(Shope, 1933). Domestic rabbit papillomas are poor producers of virus, and frequently undergo malignant transformation into carcinomas (Rous and Beard, 1935). They are of interest as an experimental model, but are not a naturally occurring tumour in the domestic rabbit (Shope 1933). As such a model, Shope papilloma virus in vivo demonstrates a malignant potential under the right genetic and environmental influences. The presence of papillomas is restricted to rabbits in states bordering the Mississippi river even though the susceptible host is spread world wide (Friedewald and Kidd, 1944). Application of carcinogenic chemicals (methylcholanthrene, tar, polycyclic hydrocarbons) at or prior to infection with virus causes a more rapid malignant transformation of papillomas in the domestic rabbit (Rogers and Rous, 1951; Rous and Friedewald, 1944).

Shope papilloma virus shows some genetic heterogeneity demonstrated by serially passaging strains in domestic rabbits (Shope, 1935). Rous (1943) suggested the presence of Shope virus variants or "cancer producing viruses" caused the malignant transformation of papillomas but this has not been supported by more recent work (Rogers et al., 1960).

1.3.7 Rabbit Oral Papilloma Virus (ROPV)

Although susceptible to experimental infection, wild rabbits have not been reported to show oral papillomatosis which occurs in domestic rabbits. The Shope virus (1.3.6) cannot induce papillomas in the oral cavity while the ROPV is strictly limited to the oral epithelium. Macroscopically and microscopically, oral papillomas are similar to and occur on the same sites as focal epithelial hyperplasia in man (section 1.3.4 (a)) (Rolzok et al.,1966; Parsons and Kidd, 1943). Infection appears to depend on animal contact especially from doe to sucklings. Virus has been recovered from mouth-washings of rabbits free from papillomas (Andrewes and Pereira, 1972). No malignant transformation has been reported.

1.3.8 Canine Papilloma Virus

A papillomavirus has been identified in one case of cutaneous papillomatosis in the dog (Watrach, 1969) and viral inclusions have been seen in the superficial epithelial layers (Allison, 1965).

1.3.9 Canine Oral Papilloma Virus

Oral papillomatosis is primarily a disease of young dogs and appears to be limited to the mucous membranes of the eye and oral cavity, though it may affect skin immediately adjacent to mucocutaneous junctions of these areas (Chambers and Evans, 1959; Chambers et al., 1960). One case has been reported of malignant transformation (Watrach et al., 1970). Other species appear to be insusceptible (Chambers and Evans, 1959 and references therein). The virus of canine oral papillomatosis is thought to be distinct from that of skin papillomatosis (Allison, 1965).

1.3.10 Equine Papilloma Virus

Skin papillomas on horses have long been recognised. The virus has been isolated and the disease transmitted to other horses. The tumours mainly involve the nose and lips of young horses but may affect other parts of the integument. The virus does not affect calves, lambs, dogs, rabbits or guinea pigs. (Cook and Olson, 1951; Bruner and Gillespie, 1973). Equine sarcoid will be discussed with Bovine Papilloma Virus (BPV) later.

1.3.11 Goat Papilloma Virus

Two reports describe papillomatosis in goats. The first by Davis and Kemper (1936) reports benign warts on anterioventral skin sites. The other describes warts on the udders of goats, some showing malignant transformation to carcinomas (Moulton, 1954).

1.3.12 Hamster Papilloma Virus (Graffi)

A virus similar in size and DNA content to polyomavirus was found in the keratinized cells of hamster papillomas of epidermal hair follicle origin (Graffi et al., 1970). On transmission to hamsters it caused epidermal papillomas, a 30 - 80% incidence of lymphomas and leukaemias within

4 - 8 weeks, and a few subcutaneous sarcomas. In rats, inoculation produced reticulum cell sarcomas while in newborn rabbits, fibromas developed at the site of inoculation. A complicating factor has been the isolation of C-type virus particles in hamster lymphomas and sarcomas induced by the Graffi virus (Graffi et al., 1963 and 1967).

1.3.13 Sheep Papilloma Virus

Benign skin fibropapillomas have been reported on the skin of sheep (Head, 1965). Attempts at transmission of virus to sheep and cattle have been unsuccessful but newborn hamsters inoculated with the virus developed fibrosarcomas (Gibbs, personal communication, cited by Barthold, 1974). Sheep inoculated with bovine papilloma virus did not develop lesions (Barthold, 1974).

1.3.14 Deer Fibroma Virus

Contagious cutaneous tumours have been reported in the deer, elk, and moose (Fay, 1970). The tumours in the deer are caused by the deer fibroma virus and are predominantly fibromatous fibropapillomas (Shope et al., 1958). There is one report of malignant transformation with pulmonary metastasis (Koller and Olson, 1971). The virus is only present in the outer epithelial portions of the tumour in deer and causes fibrosarcomas on inoculation in hamsters. No response follows inoculation of virus into horses or cattle (Koller and Olson, 1972; Tajima et al., 1968).

1.3.15 Equine Sarcoid

Equine sarcoid is a fibromatous or a fibropapillomatous tumour of the skin of horses which has long been suspected of being infectious (Ragland et al.; 1970). A papovavirus has been isolated but a virus unlike the papovaviridae has been reported from cell cultures derived from sarcoid (Allison, 1965; England et al., 1973). Variable results are reported from transmission experiments (Ragland et al., 1970). Bovine papilloma virus (BPV) causes sarcoid-like tumours in the horse (Olson and Cook, 1951) and recently, BPV specific DNA sequences have been detected in naturally occurring equine tumours (Lancaster et al., 1977).

1.3.16 Bovine Papilloma Virus (BPV)

Bovine papilloma virus has been extracted from a variety of lesions occurring on different anatomical sites in cattle. BPV was found to be the causative agent of cutaneous fibropapillomas by Creech (1929); genital fibropapillomas (McEntee, 1952); and it may be associated with chronic enzootic haematuria of cattle (Olson et al., 1965). Atypical papillomas have been shown to contain BPV (Barthold et al., 1974). BPV has been extracted from alimentary papillomas (Jarrett et al., 1978). This thesis will report the extraction and transmission of BPV from three types of teat lesions - fibropapilloma, papilloma and focal epithelial hyperplasia (Meischke, 1978).

Experimentally, BPV induces meningiomas in calves (Gordon and Olson, 1968); polypoid tumours of the urinary bladder of cattle (Olson et al., 1959); sarcoid-like lesions in the horse (Olson and Cook, 1951); fibroblastic tumours in the hamster (Boiron et al., 1964; Cheville, 1966; Lasneret et al., 1965; Robl et al., 1972; Robl and Olson 1968), and fibromatous tumours in C3H/eB mice (Boiron et al., 1964).

1.3.17 Other Papilloma Viruses

Cutaneous papillomas have been reported on Cebus (Lucke et al., 1950) and Rhesus (Smith et al., 1972) monkeys. The former was transmissible to old and new world monkeys. Virus has not been isolated, but papillomas have also been reported on Macaca (Brown et al., 1972) and Colobus (Boever and Kern, 1976) monkeys.

Papovaviruses have been seen by EM from papillomas on Virginia Opossum (Koller, 1972); and on the chaffinch and related species (Osterhaus et al., 1977). A transmissible genital papillomatosis occurs in pigs (Parish, 1961 and 1962). Papillomas found on fish appear to be associated with a virus other than the papova group (Wellings and Chuinard, 1964) with the possible exception of the epidermal papillomas in Salmon (Chronwall, 1976).

1.3.18 Summary - Papovaviruses in vivo

Table 7 lists the papovaviruses, their natural hosts and their oncogenicity under natural and experimental conditions.

TABLE 7: Papovaviruses in vivo - host range and oncogenicity

Virus	Oncogenicity in Natural Host	Experimental Oncogenicity
Polyoma	carcinomas, adenomas, sarcomas in mouse	hamster: sarcomas and meningiomas; sarcomas and fibromas in rats, rabbits, mastomys, ferrets and guinea pigs
SV40	fibrosarcomas in monkeys	central nervous system fibroblastic neoplasms in hamsters and mastomys. lymphosarcoma and reticulum cell sarcomas in weaning hamsters
BKV, RFV MMV	associated with fibroblastic neoplasms in the central nervous system of man. rarely, affects urogenital epithelium	produce fibroblastic neoplasms of CNS and elsewhere in newborn hamster
JCV	associated with PML and possibly glioblastomas in man	newborn hamster-differential oncogenicity between virus strains. pineocytomas and fibroblastic neoplasms of brain. Sarcomas in non CNS sites
K Virus	pneumonitis and liver damage in newborn mice, not oncogenic	not oncogenic
Porcine Papovavirus	not oncogenic in pigs	not known
RKV	not oncogenic in rabbits	not pathogenic or oncogenic
HDV	not known	not known
STMV	not known	not known

TABLE 7: (Cont'd) Papovaviruses in vivo - Host Range and Oncogenicity

Virus	Oncogenicity in Natural Host	Experimental Oncogenicity
HPV	Focal epithelial hyperplasia, benign papillomas and under certain conditions, carcinomas	May be transmissible to dogs. producing genital papillomas
Shope	papillomas and carcinomas under certain conditions in wild rabbit.	papillomas and carcinomas in domestic rabbit
ROPV	benign papillomas in domestic rabbit	not known
Canine Skin P. V.	benign papillomas in dog	not known
Canine Oral P. V.	oral papillomas in dog rarely carcinomas	other species appear to be insusceptible
Goat Skin P. V.	cutaneous papillomas occasionally carcinomas in goat	not known
Graffi	papillomas in hamsters	newborn hamsters - subcutaneous sarcomas; fibrosarcomas in rabbits; reticulum-cell sarcomas in rats
Sheep P. V.	fibropapillomas in sheep	fibrosarcomas in hamsters
Deer Fibroma Virus	cutaneous fibromas, fibropapillomas rarely malignancies	fibrosarcomas in hamsters
BPV	cutaneous papillomas, fibropapillomas alimentary papillomas teat papillomas fibropapillomas and focal epithelial hyperplasia alimentary carcinomas, adenocarcinomas under certain conditions	meningiomas in calves; urinary bladder polyps in cattle; sarcoid-like lesions in horse; fibroblastic tumours in hamster and mouse.

1.4 Papovaviruses In Vitro

Most members of the papovaviridae have the ability to transform cells in vitro. A number will do so with just their DNA - in some cases this is more efficient since the capsid proteins may hinder DNA entry into the cell, while in other cases, DNA induced transformation is far less efficient than whole virus transformation.

When papovaviruses infect cells, in vivo or in vitro, two modes may result, virus production or cellular transformation. Cells which produce virus are called permissive. Cells which transform do not normally release virus and in some cases have never been shown to release virus. Table 8 lists the characteristics of transformed cells. Some transformed cells display all the characteristics listed but more commonly papovavirus transformed cells only display some of them.

TABLE 8: PRINCIPAL CHARACTERISTICS OF TRANSFORMED CELLS

(source Harnden, 1976)

1. Variability in morphology of individual cells
2. Tendency to grow in a randomly oriented manner even when in contact with other cells.
3. Tendency to form multiple layers of cells.
4. Low requirement for serum in the medium
5. Ability to form colonies in soft agar
6. Indefinite lifespan in culture
7. Presence of new surface and/or nuclear antigens
8. Altered distribution of binding sites for plant lectins
9. Presence of chromosome abnormalities
10. Ability to produce tumours on inoculation with appropriate host.

1.4.1 Polyoma and SV40 In Vitro

Polyomavirus will transform mouse, rat, rabbit, guinea pig, dog; cattle, hamster, monkey and human cells in vitro. Infectious virus cannot normally be recovered from cells without the aid of agents such as Ultra-Violet (UV) light or mitomycin C or the fusion of transformed cells to cells permissive for replication (Black, 1968; Fogel and Sachs, 1969). Polyoma

virus normally only replicates in cultures of mouse origin and occasionally in rat tumour tissue cultures (Andrewes and Pereira, 1972). In permissive cell monolayers, polyoma causes plaques due to lytic infection. Naturally occurring plaque variants have been described causing different plaque types.

SV40 can transform pig, rabbit, mouse (Black and Rowe, 1963), rat (Diderholm et al., 1966), monkey (Fernandes and Moorhead, 1965) and human cells (Shein and Enders, 1962; Koprowski et al., 1962) under in vitro conditions. Following exposure of human cells to SV40 both transformation and virus replication occur and so the cells are called semipermissive for SV40. SV40 replicates most efficiently in monkey cell cultures.

Both polyoma and SV40 have naturally occurring and experimentally induced mutant strains. Temperature sensitive mutants which affect most if not all genes required for lytic infection have been isolated. These mutants are selected on the basis of effects in lytic infection at the restrictive temperature. Virus replication can be defective at a number of stages, determined by measuring the induction of cellular DNA synthesis, viral DNA synthesis and the appearance of viral antigens or particles.

Host range mutants are defective in viral functions required for transformation. They were originally selected for their ability to grow on transformed mouse cells. So far more than a dozen host range mutants have been shown to be unable to transform mouse, rat or hamster cells.

Defective mutants have deletions, insertions, substitutions or other rearrangements in their DNA. Generally they will not replicate without the use of a nondefective helper virus or DNA.

Adenovirus-SV40 hybrid viruses are adenoviruses which contain covalent genome insertions of SV40 DNA. They were originally isolated because human adenoviruses do not replicate efficiently in monkey cells unless the cells are coinfected with SV40. The SV40 DNA insertions exert a helper function rendering the hybrid virus non-defective for replication in monkey cells. Different antigens are expressed in cells infected by the various hybrids suggesting that two or more parts of the SV40 DNA may be involved.

A number of reviews on SV40 and polyoma in vitro and the genetics of these viruses have been published recently (Eckhart, 1977; Fried and

Griffin, 1977; Khoury and Salzman, 1975; Williams, 1976; Rowson, 1976).

1.4.2 BKV (RFV, MMV) in vitro

Table 9 lists the cells known to be permissive for BKV replication. BKV produces cytopathic effects (CPE) but no progeny in primary Rhesus monkey kidney cells (Gardner et al., 1971), primary African green monkey cells, and two cell lines derived from the latter (Takemoto and Mullarkey, 1973).

TABLE 9: CELLS PERMISSIVE FOR BKV REPLICATION

Source	Cell Type	References
Human	primary foetal kidney	Dougherty and Di Steffano, 1974
	primary foetal fibroblast	Portolani et al., 1975
	primary foetal liver and lung	Seehafer et al., 1975
	primary foetal brain (glial)	Lecartas et al., 1974;
	WI - 38 cell line	Takemoto and Mullarkey, 1973
	foetal kidney	Dougherty and DiSteffano, 1974
	foetal lung	Coleman et al., 1973
	foetal brain	Takemoto et al., 1974
Monkey	VERO (African green monkey kidney)	Takemoto and Mullarkey, 1973

Table 10 list the cells that have been shown to be transformation sensitive to BKV infection.

TABLE 10: CELLS THAT ARE BKV TRANSFORMATION SENSITIVE

Source	Cell Type	References
Hamster	primary foetal fibroblast	Dougherty, 1976
	primary foetal kidney	Portolani et al., 1975
	BHK -21 (hamster kidney)	Major and DiMayorca 1973
	brain	Tanaka et al., 1976
Rat	primary kidney cells	Vander Noorda 1976
Rabbit	primary kidney	Mason and Takemoto, 1977
Nude Mouse	kidney	Costa et al., 1977

Some BKV transformed cells (e.g. hamster kidney) are tumorigenic in vivo while others (e.g. rabbit primary kidney) are not. Different BKV related antigens are expressed in different transformed cells and some authors suggest that differing BKV strains (MMV, RFV) may vary biologically (Mason and Takemoto, 1977). Transformation with BKV DNA of hamster and rat cells has been achieved with greater frequency and shorter time than with intact virus (van der Noordaa ,1976). This suggests that BKV virions do not become uncoated or penetrate efficiently in rodent cells (Takemoto and Martin, 1976).

1.4.3 JCV in vitro

In contrast to BKV, SV40 and polyoma, JCV has an extremely restricted cell range. JCV grows and produces CPE only in primary foetal human glial (PHFG) cell cultures that contain a high proportion of spongioblasts. It may take 6 - 9 months post infection for CPE to be observed in PHFG using JCV from diseased brain. JCV is strongly cell associated and cultures require mechanical disruption or receptor destroying enzyme (RDE) to free virus from cell debris. After serial passage in culture, CPE appear 12 - 14 days post inoculation (Padgett and Walker, 1976). Padgett et al., (1977) attempted to infect a wide range of cell types from human, primate, hamster, mink and mouse origins without success either in the form of JCV replication, CPE or transformation.

1.4.4 Other Polyomaviruses in vitro

a) The K-virus of mice has been shown to cause transformation in embryonic mouse lung, 6 weeks post infection. One line of transformed cells has produced tumours in newborn or irradiated mice (Takemoto and Fabisch, 1970, cited by Andrewes and Pereira, 1972).

b) Rabbit Kidney Vacuolating Virus (RKV)produces cell-vacuolation in primary rabbit kidney cell cultures, CPE form on monolayers but cells of other species appear insusceptible (Hartley and Rowe, 1964). Mason and Takemoto (1977), report growth of RKV on BKV transformed rabbit kidney cultures and used the technique to plaque titrate RKV.

c) H D virus has been reported to transform the African green monkey kidney cell line, VERO. (Waldeck and Sauer, 1977).

d) The owl monkey papovavirus has been reported to infect only owl monkey kidney, cornea and iris cell cultures (Daniel et al., 1976).

e) STMVirus has been reported to replicate and produce CPE in primary kidney cultures from the stumptailed Macaque and Rhesus monkeys. STMV is antigenically related to SV40 and BKV but no reports of in vitro transformation have been made. (Reissig et al., 1976).

1.4.5 Human Papilloma Virus (HPV) in vitro

A number of reports claim to have grown HPV in tissue culture. Mendelson and Kligman (1961) inoculated monkey kidney cell cultures which after the third passage, six weeks post inoculation, showed CPE. Inoculation of ultrafiltrates of tissue culture fluid into twenty human subjects and observed the development of verruca at the inoculation site in 50% of subjects 3 to 12 months post inoculation. Subsequent reports by Macpherson (1962); Morgan and Balduzzi (1964); Oroszlan and Rich (1964); and Noyes (1965) have not been confirmed (Butel, 1972). Furthermore, when virus containing verrucae are grown in vitro, evidence of viral replication by electron microscope and immunohistological methods is lost (Nirmura et al., 1975).

The earlier observation of Butel (1972) that HPV infected confluent monolayers of human embryonic kidney cells show a stimulation of DNA synthesis, was supported by Lancaster and Meinke (1975) who reported HPV DNA persistence in cultures of a human foreskin derived cell line.

Eisinger et al., (1975) reported HPV induced replication and CPE in a human epithelial cell line (B.E.). They confirmed this using immunological, EM and radio-labelling techniques. In another report of the same experiments, Hadden (1975) emphasised that HPV production was low using BE cells and that a search for more productive cells had commenced. As with the earlier reports, these findings await confirmation.

Only one report describes HPV induced transformation of primary human foetal skin and muscle cultures (Noyes, 1965), but this has not been supported by other authors.

1.4.6 Shope Papilloma Virus in vitro

Shope virus has never been grown in vitro. No changes were reported in cells from rabbit skin to which virus had been adsorbed, but these gave rise to papilloma when transferred to hamster cheek pouches. Nuclear virus specific antigen disappeared from papilloma derived cells held at 37°C but reappeared at 30°C in the cytoplasm (Shiratori et al., 1969).

Various authors have studied in vitro cultivated cells from Shope induced papillomas (Coman, 1946; Ishimoto et al., 1970; Ishimoto and Ito, 1971) which do display certain antigenic similarities seen in in vivo lesions. In vitro infection of rabbit cells with Shope virus has also been studied and it has been shown by immunofluorescence that virus does infect but not replicate in cells (De Maeyer, 1962; Kreider et al., 1967; Moulton and Lau, 1964). Shope virus infected cells do not show morphological changes but the virus is able to alter the permissivity of rabbit cells to SV40 penetration (Chardonnet et al., 1976).

1.4.7 Bovine Papilloma Virus (BPV) in vitro

BPV has been found to transform bovine kidney and foetal bovine conjunctiva (DBC) cell lines, secondary cultures of foetal bovine heart (Black et al., 1963), foetal bovine skin cells, primary cultures of foetal bovine skin pieces, secondary fibroblastic cultures of various strains of foetal mouse (Thomas et al., 1963 & 1964), and foetal hamster cell cultures (Geraldes, 1969). BPV DNA has also been shown to transform foetal bovine skin cells (Boiron et al., 1965).

Olson et al., (1960) claimed to have grown BPV in the chorioallantoic membrane (CAM) of fertile chicken eggs. These results await confirmation. (See also Bagdonas and Olson, 1954). Olson et al., (1960) used BPV grown on chicken CAM to vaccinate calves but without success. This thesis will report transformation of foetal bovine cultures of various tissues and attempts to cultivate BPV in vitro.

1.4.8 Other Papilloma Viruses in vitro

There are no reports in the literature of replication, transformation or CPE formation in vitro by any of the other papilloma viruses listed in

section 1.3 of this thesis. This is certainly partly because, as with the newer polyomaviruses, insufficient research has been completed. Strong candidates for in vitro transformation of host cells must include the deer fibroma virus and the sheep fibropapilloma virus. Like many earlier reviewers (Rowson and Mahy, 1967) this author is tempted to conclude that no papillomavirus has been cultivated in vitro. However, the reports of successful HPV or BPV (the two most studied papilloma viruses) replication in vitro are many, are spread over 15 years and come from widely varied sources. The probable conclusion is that HPV and BPV have indeed been cultivated in vitro, but inefficiently and under exceptional conditions.

1.5 Immunological Aspects of Papovavirus Infection

Papovaviruses themselves are composed of major capsid protein (VPI); two minor structural proteins (VP2 and VP3), at least four different host derived histones which wrap the viral genome and the supercoiled DNA of approximately 5,000 base pairs. Cell infection by virus is accompanied by changes on the cell surface and within the cell cytoplasm, nuclear membrane and nucleus. When virus or transformed cells are inoculated into appropriate hosts, they may immunise the host against further inoculations. Exactly which antigens are expressed by virus infected cells depend largely on the degree to which infection has progressed to the endpoints of replication or transformation, but also on the host response to these changes. Those viruses most easily cultivated in vitro are also those about which the most immunological information is available. This review will only briefly outline the major features of the polyomavirus group related antigens before pursuing the papillomaviruses in greater detail.

1.5.1 Polyomaviruses - Immunological Aspects

It is possible to produce specific antisera against the whole virus, and this is called the Virion (V) antigen. It is now generally agreed that the V-antigens are the major structural proteins. V-antigens are detected in the nuclei of permissive cells late in the lytic cycle, following viral DNA replication, by immunofluorescent staining (Mayor et al., 1962). Since it is likely that the capsid protein is synthesised in the cytoplasm, its presence in

the nucleus is probably due to rapid transport for virus assembly. It is possible, however, that V-antiserum is specific for assembled coat protein(s) which may be present only in the cell nuclei (Khoury and Salzman, 1975).

Unlike the V-antigen, the intranuclear tumour (T) antigen appears early following virus infection (10 - 18 hours - Rapp et al., 1964; Hoggan et al., 1965). While it was first detected using a complement fixation (CF) method, (Takemoto and Habel, 1965), it can be localised in the nucleus using immunofluorescent (IF) techniques (Rapp et al., 1964). Antibodies for the detection of T-antigen are present in sera from animals bearing SV40 or polyoma tumours. T-antigen is heat labile; sensitive to trypsin but not to DNA'se (Gilden et al., 1965). Its synthesis is not affected by inhibitors of DNA replication (Gilden et al., 1965) but is affected by actinomycin D, interferon (Oxman et al., 1967) and cycloheximide (Gilden and Carp, 1966). This suggests that de novo transcription and translation of viral DNA are required for T-antigen production. Cell-free translation of SV40 early messenger RNA coding for viral T-antigen (Prives et al., 1977) has led to the identification of SV40 T-antigen (Robb, 1977) as a protein with a molecular weight of about 90 - 100,000. Study of SV40 host range mutants (section 1.4.1) which are no longer able to transform cells to anchorage independent growth has led to the identification of little t antigen (a protein of molecular weight 15 - 20,000) (Prives et al., 1977; Crawford et al., 1978). Recent evidence points to the fact that T and t are immunologically related and nearly identical biochemically (Fiers et al., 1978), with t representing a truncated form of T.

T-antigen has been shown to be a prerequisite for SV40 replication; is required for the initiation and the maintenance of transformation; it is also an autoregulator in that it shuts off its own synthesis at the level of transcription (Fiers et al., 1978, and references therein). Both T and t are coded for in the early region of SV40 DNA (Fiers et al., 1978) however the appearance variability of T-antigen does depend in part on host cell parameters (Kaplan et al., 1976).

The U-antigen has been detected by CF and IF methods in SV40-infected and transformed cells; is found at the nuclear membrane and is heat stable (Lewis and Rowe, 1971). U-antigen reacts with serum from

tumour bearing hamsters. T and U-antigens were thought to differ at least in part, because a few batches of anti T sera did not contain anti U antibodies. Furthermore an adenovirus SV40 mutant (Ad $2^{+}ND_{,}$) induces U antigen but not T antigen (Khoury and Salzman, 1975). Recently it has been shown that T and U antigens are both encoded in the early region of SV40 DNA and are now known to be the same molecular species. It is possible that U antigen is in fact T-antigen localised differently intracellularly (Robb, 1977).

Tumour-Specific Transplantation Antigens (TSTA) are the least well understood of the virus related neo-antigens. They can be studied in vivo by transplantation rejection (Coggin et al., 1969) or in vitro where they are localised at the cell surface (Habel, 1961; Sjogren et al., 1961; Habel and Eddy, 1963; Koch and Sabin, 1963; Defendi, 1963). TSTA production can be blocked with inhibitors of protein synthesis and DNA-dependent RNA synthesis suggesting that TSTA is a virus-induced protein during productive infection (Girardi and Defendi, 1970). TSTA was partly purified by Chang et al., (1977); it has been found in nuclear preparations of SV40 transformed cells (Anderson et al., 1977) and binds to cellular DNA (Chang et al., 1977). There is a suggested homology between TSTA and T-antigen and there appear to be two roles for this protein, that of a tumour rejection agent in vivo and a regulatory protein for the growth of cells in vitro. (Chang et al., 1977).

Finally, there are several other virus induced cell related antigens which have been detected; cytoplasmic C-antigen (Tilz et al., 1969); cell surface SV40 specific graft rejection antigen-TATA (Duthuherbet et al., 1973); the tumour associated cell surface antigen - TSSA which is identical with the S-antigen detected by membrane immunofluorescence and is probably identical to TSTA antigen (Kato, 1977). These three, TSTA, S and TSSA may in fact be fetal antigens switched on by the virus (Kato, 1977).

The interrelationships between the polyomaviruses have been discussed earlier (see sections 1.1.2 and 1.2.3) and at least polyoma, SV40, BKV (RFV and MMV), JCV and STMV are similar antigenically in some respects and different in others.

While not conclusively proved in all SV40 polyomavirus-cell systems, it is probable that two or three main antigens exist in virus permissive or transformed cells; the V-antigen; the nuclear T-antigen (same as U); and

the TSTA antigen (likely to be the same as TSSA and S antigen). It is quite possible that T and TSTA antigens are homologus (Chang et al., 1977). The tentative conclusion may be that virus replication or transformation results in two main antigenic changes. Firstly virus components in the nucleus, which appear in their final form as V-antigens but at various intermediate stages are complexed with or are identical to foetal antigens expressed as a result of the initial infection. That the situation is so complex,is due to:(1) the variety of techniques used (2) the varying degree to which viruses proceed to replication of transformation in different cell systems (3) the use of differing terms for the same phenomena and (4) the inability, until very recently, to characterise the antigens being detected.

1.5.2 Human Papillomavirus - Immunological Aspects

Pass et al., (1971) have demonstrated nuclear and cell surface antigens in human wart tissues detected by indirect IF using antisera prepared in rabbits to homogenates of wart tissue. The two antigens were isolated from tissue homogenates by elution from antibody-sepharose immuno-adsorbents and neither was found to be a structural component of HPV. The antigens were detected in warts, squamous cell carcinomas, foetal skin and in psoriatic epidermis, but not in basal cell carcinomas or normal skin (Pass and Marcus, 1973). These antigens correspond to the T or TSTA antigens seen following infection by polyomaviruses (section 1.5.1).

Virus antigens have also been detected. Almeida and co-workers first observed humoral responsesto HPV (Almeida and Goffe, 1965; Goffe et al., 1966). Genner (1971) and Ogilvie (1970) confirmed the presence of antibodies to V-antigen but were unable to correlate wart regression with antibody titre using complement fixation and passive haemagglutination techniques. These authors reported that the anti-V antibody was largely in the form of IgM and considered it ineffective in producing regression or protection to HPV infection (Goffe et al., 1966). Pass and Maizel (1973) found that anti-V antibody was directed toward the major structural protein (VP1) of the viral capsid. V-antigen can be demonstrated in the nuclei of verrucae (Walter et al., 1965). More recently, regression of verrucae has been associated with anti-V IgG immunoglobulins, however not all verrucae contain

V-antigen (Matthews and Shirodaria, 1973; Shirodaria and Matthews, 1975). Pyrhonen and Johansson (1975) suggested that the cure for verrucae was related to IgG anti-V antibodies which they detected using immunodiffusion (ID) and complement fixation (CF).

Using ID, no antibody - antigen reaction was detected using condyloma extracts, however counter-immunoelectrophoresis did detect anti-condyloma antibodies but these were apparently not related to condyloma regression (Abcarian and Sharon, 1977). Nevertheless, the use of autogenous vaccine did result in the rapid regression of condyloma in 80% of patients (Abcarian and Sharon, 1977 ; Abcarian et al., 1976). The use of autologous vaccines in the treatment of juvenile laryngeal papillomatosis has also met with considerable success (Pinson et al., 1976).

Clinical investigations report regression of warts after inflammation (Tagami et al., 1974); after treatment with a transfer factor (Stevens et al., 1975); di-nitrochlorobenzene (DNCB); and a variety of immune adjuvants (Lewis, 1973; Grunberg et al., 1973). In renal transplant patients or patients with long lasting cell-mediated immunodeficiencies, warts are numerous and rarely regress (Thivolet et al., 1975; Spencer and Anderson, 1970; Reid et al., 1976; Johansson et al., 1977). The increased prevalence of warts in patients with systemic lupus erythematosis (SLE) is inversely correlated with rheumatoid factor activity and prevalence of anti-V antibodies (Johansson et al., 1977).

Delayed hypersensitivity skin reactions (DHR) to wart tissue extracts (Maderna, 1934) and purified HPV have been reported. These were most frequent in patients with regressing or "past" warts (Viac et al., 1977). The incidence of anti-V antibodies was higher in patients with a positive DHR following test, suggesting a "booster" effect.

Cell mediated immunity (CMI) is likely to be an integral part of the host defence to warts (Lee and Eisinger, 1977). CMI to HPV and tissue extracts can be measured using lymphocyte transformation (LT) (Lee and Eisinger, 1977) and leucocyte migration inhibition factor (LMIF) (Morrison, 1974 and 1975b). CMI is short lived and could only be demonstrated in patients with warts of less than 1 year duration. Lee and Eisinger (1977) consider that the lack of correlation of LT and LMIF results using tissue extracts compared with purified HPV indicate the presence of non-V antigens in wart extracts.

Morrison (1975a) found no correlation between CMI and anti-V antibodies.

The existence of several types of HPV has been discussed earlier (see section 1.1.2 and 1.3) and Pfister and Zur Hausen have recently demonstrated seroepidemiological differences in the prevalence of anti-V antibodies to two HPV types (Pfister and Zur Hausen, 1978).

In conclusion, HPV is similar to the polyomaviruses (Section 1.5.1) in producing both V-antigens and HPV-induced cell associated antigens (T or TSTA).

1.5.3 Shope Papilloma Virus (SPV) - Immunological Aspects

In vivo and in vitro, SPV has been shown to produce three antigens. The V-antigen has been demonstrated within papilloma cells in vivo (Noyes and Mellors, 1957), in cells infected in vitro but not transformed (Osato and Ito, 1968) and papilloma derived cells grown in vitro (Ito, 1970; Shiratori et al., 1969). Furthermore, considerable evidence now supports the presence of SPV associated nuclear T-antigens in vivo (Zibler, 1958) and in SPV infected cells in vitro (Yoshida and Ito, 1968). Another SPV-induced event is the appearance of surface antigen (Ishimoto and Ito, 1969 and 1971) detected by IF on unfixed cultured cells derived from Cottontail papilloma tissue. Like the polyomaviruses and HPV, SPV induces both V-antigens and cell associated antigens (T or TSTA).

1.3.4 Bovine Papilloma Virus (BPV) - Immunological Aspects

V-antigen has been detected in fibropapillomas using IF (Smithies and Olson, 1961). Quantitation of BPV and anti-V antibody (Koller et al., 1974) using immunodiffusion was used to investigate the regression of fibropapillomas in relation to anti-V antibody in the serum. No correlation was found between fibroma regression and anti-V antibody and fibropapillomas continued to grow progessively (Barthold and Olson, 1974a).

The fibroma cells of BPV induced fibropapillomas lack V-antigens detectible by IF and EM (Smithies and Olson, 1961; Tajuna et al., 1968; Robl and Olson, 1968) but possess a surface antigen detected by indirect IF on live unfixed BPV induced fibroma cells grown in vivo and in vitro. (Barthold and Olson, 1974b). Serially sampled fibropapillomas show considerable

histopathological evidence of a major role played by CMI in the regression of at least the fibroma component of bovine fibropapillomas (Barthold and Olson, 1974b).

Bovine papilloma virus is thus similar to SPV, HPV and the polyoma-viruses in its capacity to induce V-antigen and cell associated antigens. Although no nuclear T-antigen has been demonstrated in BPV induced tumours, this may be due to insufficient research since these have been found in HPV and SPV infected cells.

1.5.5 Other Papillomaviruses - Immunological Aspects

A V-antigen has been detected in canine oral papillomas and anti-V antibody in dogs with regressing tumours and the likelihood of a cell associated nonvirion antigen is discussed by Chambers et al., (1960). Other papillomaviruses have not been studied insufficient depth to provide useful comparative data.

1.5.6 Summary - Immunological Aspects of Papovaviridae

The response of the host to papovavirus infection is complex and still poorly understood. It doubtlessly involves at least three main defence mechanisms: anti-virus antibody; anti-cell antibody and cell mediated immunity. The relative contribution of each is unknown although most authors postulate that all three are required for effective rejection of existing infection and resistance to challenge.

1.6 Physicochemical Characteristics of the Papovaviridae

Mention has been made in earlier sections of this review of the chemical composition of papovaviruses and these features will not be discussed in great detail here. Three physicochemical aspects are however, important. They are: (1) ability to agglutinate red blood cells; (2) virus particle diameter, and (3) virus thermostability. Their importance is due to the fact that differences in these characters are often used to differentiate between individual papovavirus members. Indeed, virus size and the related parameters of virus and DNA molecular weight are one of the main characters used to separate the polyomaviruses from the papillomaviruses. Thermo-

stability is of importance, especially with BPV, since milk for human consumption is likely to be contaminated with BPV from teat papillomata. Haemagglutination has until very recently represented one of the principal methods of quantitating virus and anti-V antibodies.

1.6.1 Papovavirus Haemagglutination

Polyoma virus agglutinates red blood cells (RBC) of many species (Sachs et al., 1959) but guinea pig RBC are commonly used. It attaches to RBC by the same mucoprotein receptors as influenza virus. These receptors can be destroyed by influenza virus or receptor destroying enzyme (RDE) from Vibrio comma, but polyoma itself does not destroy the receptors (Hartley et al., 1959). Treatment of tissue cultures with RDE reduces their susceptibility to polyoma virus infection suggestions that the receptors are involved in the attachment or entry of virus into cells (Rowe, 1961). The haemagglutinin is present on both full and empty particles and is readily eluted from the erythrocyte receptors without destroying them at 37°C (Crawford, 1969). The haemagglutination assay for virus or antibody must therefore be carried out at 4°C (Rowson, 1976).

The K-virus of mice haemagglutinates sheep RBC at room temperature or 37°C. Heating of the virus suspension at times unmasks the haemagglutinin (Kilham, 1961).

RKV of rabbits agglutinates guinea pig RBC at 4°C and 20°C and like polyoma, receptors are destroyed by RDE (Hartley and Rowe, 1964).

Shope papilloma virus does not haemaggluntinate RBC, however rabbit RBC do adsorb the virus (Andrewes and Pereira, 1972).

While SV40 does not agglutinate RBC, the closely related BKV does agglutinate human, guinea pig and chicken RBC (Gardner et al., 1971; Mantyjarvi et al., 1973). Maximum HA is achieved with human O RBC at 4°C in buffers pH6.1 - 7.4 with titres about fourfold greater than with guinea pig RBC (Mantyjarvi et al., 1973; Seehafer et al., 1975). The virus associated haemagglutinin is resistant to ether; heating at 56°C for 30 minutes; chloroform; and 0.1% β - propiolactone. No HA is observed at 37°C (Dougherty and DiStefano, 1974; Pitko et al., 1975; Takemoto and Mullarkey, 1973). The human RBC HA receptors were reduced but not destroyed by RDE.

The inhibitors of BKV HA are present in sera of many species and these may be removed using RDE, acetone and sodium iodide (Padgett and Walker, 1976).

Like BKV, JCV agglutinates human, guinea pig and chicken RBC at 4°C (Padgett and Walker, 1973; Walker et al., 1974). The agglutinin is virus associated (Osborn et al., 1974). Sera of many species contain HA inhibitors and sodium iodide is used to remove them (Padgett and Walker, 1973).

BPV has been shown to agglutinate mouse RBC at 4°C between pH6.8 and 8.4. BPV is eluted at 37°C like polyoma and BKV. RBC receptors are resistant to RDE and Influenza neuraminidase. HA is associated with both full and empty particles (Favre et al., 1974).

1.6.2 Papovavirus Particle Diameter

Table 11 lists the wide range of estimates of virus particle diameter using both negatively stained and thin section electron microscopy. It should be noted that each technique has its' own bias. Negatively stained preparations may contain flattened virions and also a protein coat may on occasions lead to a larger apparent size. Thin section diamter estimates may be smaller because of appreciable shrinkage due to dehydration and embedding (Gross, 1970).

The estimates vary considerably with the following ranges according to Table 11: Polyoma - 2mμ; SV40 - 5mμ; BKV - 3.5mμ; JCV-5.5mμ; K - 10mμ; STMV - 5mμ; HPV - 27mμ; SPV - 34mμ; ROPV - 12mμ; Canine OPV - 10mμ; Deer FV - 18mμ; BPV - 19mμ. While there are wide differences likely to be caused by preparatory methods (see earlier) it is surprising to notice that the variation in diameter estimates of the polyomavirus group range 2 - 10mμ and the papilloma virus group range 10 - 34mμ. Mention has already been made of the fact that different types of HPV occur (section 1.3) and though no evidence appears in the literature, it may be that diameter measurements of single types will be different to others. In this context the polyomaviruses of monkeys, STMV, OMPV and SV40 are from 30 - 45 mμ, a range of 15mμ; alternatively the polyomaviruses of mice, polyoma and K-virus are from 40 - 50mμ, a range of 10mμ.

In summary, if one classifies papovaviruses according to species of

TABLE 11: PAPOVAVIRUS PARTICLE DIAMETER.

Virus	Prep*	Diameter (mμ)	References
Polyoma	U	45 (1); 43 (2)	1.Andrewes and Pereira, 1972
SV40	N	42.61 (3)	2.Rowson, 1976
	U	40-45 (4)	3.Bahr et al., 1976
BKV	N	38-43.5 (5)	4.Anderer et al., 1967
JCV	N	38-43.5 (5)	5.Padgett and Walker, 1976
	T	39.1 (5)	6.Reissig et al., 1976
K-virus	U	40-50 (1)	7.Daniel et al., 1976
RKV	T	47 (1)	8.Melnick et al., 1974
STMV	N	40-45 (6)	9.Williams et al., 1961
OMPV	T	33 (7)	10.Melnick et al., 1952
HPV	N	55 (8); 55 (9); 52 (10).	11. Gross, 1970
	T	40-54(11);33(12);38(13)	12.Charles, 1960
HPV(FEH)	T	40-60 (14)	13.Bunting, 1953
SPV	N	56-60 (15); 40-47 (11)	14.Praetorius-Clausen and Willis, 1971
	T	26-29 (16); 33 (17)	15.Chambers and Ito, 1964
ROPV	N	50-52 (18)	16.Haguenan et al., 1960
	T	40 (18)	17.Moore et al., 1959
Canine OPV	N	40-50 (19)	18.Rozok et al., 1966
Deer FV	N	50-53 (20)	19.Cheville and Olson, 1964a
	T	35 (20)	20.Tajima et al., 1968
FPV	N	51.7± 1.7 (21)	21.Albert et al., 1977
BPV	N	47.5 (22); 47(23);51-54(20)	22.Huck, 1965
	T	35 (20)	23.Levy et al., 1963

* Prep = Preparatory method - N= negative staining; T = thin section; U = unspecified.

origin, particle diameter estimates range, in the polyoma group 3.5 - 15mμ and the papilloma group 10 - 34mμ. The practice of differentiating the papovaviruses on virus size giving polyomaviruses a general range 43 - 45mμ and papillomaviruses 52 - 54mμ (Andrewes and Pereira, 1972) is not supported by the literature which shows substantial overlap between the groups.

1.6.3 Thermostability of Papovaviruses

Table 12 summarises the literature on the thermostability of the papovaviridae. Most members are able to survive at least 30 minutes at 60°C, the equivalent of pasteurisation. It is of interest that the DNA is able to withstand high temperatures for long periods largely because of its' unique, closed, circular form. At high temperatures the DNA disassociates into single strands but reassociates on cooling (Crawford, 1964). The increased thermostability of viral DNA over whole virus may be due to the earlier denaturing of viral capsid protein.

There is no reference in the literature on the effects of time-temperature on BPV. This thesis will report on this aspect because of its potential importance in three areas (1) animal to animal transmission of teat papillomatosis via heat treated milking devices (2) adult to calf transmission using pasteurised milk (3) human exposure to BPV via pasteurised milk.

1.7 Summary of the Literature Review

Table 13 summarises the main effects of the papovaviridae *in vivo*; Table 14, the main *in vitro* effects; and Table 15, immunological and physicochemical characteristics. While more research is needed, it is apparent that in most characteristics, the polyoma and papillomaviruses overlap considerably, often making the separation of the papovaviridae into two groups more confusing than helpful. The major difference remaining between the two groups is that there is at present no reliable method of growing papillomaviruses *in vitro*. It is probable that this is due to the absence of a suitable cell system, since some polyomaviruses have extremely restrictive growth requirements.

TABLE 12: TIME-TEMPERATURE INACTIVATION OF PAPOVAVIRUSES

Virus	Time (min)	Temperature(°C)	Effect*	References
SPV	30	67	NI	Fischer and Green, 1947
	30	70	I	
ROPV	30	65	NI	Parsons and Kidd, 1943
	30	70	NI	
HPV	30	50	NI	Rowson and Mahy, 1967
Canine Oral PV	60	45	NI	Andrewes and Periera, 1972
	60	58	I	
Equine Skin PV	30	55	I	Cook and Olson, 1951
Polyoma	30	60	NI	Bradsky et al., 1959
	30	70	I	
K-virus	180	70	NI	Fischer and Kilham, 1953
	270	70	I	
	240	60	NI	
SV40	60	46	NI	Mayor et al., 1963
RKV	30	60	NI	Hartley and Rowe, 1964
	30	70	NI	
JCV	60	50	NI	Padgett et al., 1977
SPV DNA	80	70	NI	Ito, 1975
	180	70	NI	
	360	70	NI	

* Effect: NI - not inactivated
I - inactivated.

TABLE 13: THE PAPOVAVIRIDAE IN VIVO

Tumour type	Benign Epithelial	Benign Mesenchymal	Carcinoma	Adenoma	Sarcoma	Other
VIRUS						
Polyoma	+	+	+	+	+	+
SV40	-	+	-	-	+	+
BKV	-	+	-	+	+	-
JCV	-	+	-	-	+	+
STMV	-	-	-	-	-	-
OMPV	-	-	-	-	-	-
HDV	-	-	-	-	-	-
K-virus	-	-	-	-	-	-
RKV	-	-	-	-	-	-
HPV	+	-	+	-	-	-
SPV	+	-	+	-	-	-
ROPV	+	-	-	-	-	-
Canine PV	+	-	-	-	-	-
Canine OPV	+	-	-	-	-	-
Equine PV	+	-	-	-	-	-
Goat PV	+	-	+	-	-	-
Hamster PV	+	+	+	-	+	+
Sheep PV	+	+	-	-	+	-
Deer FV	+	+	-	-	+	-
Equine Sarcoid	+	+	-	-	-	-
BPV	+	+	+	+	-	-

Note: + - reported to occur in the literature
- - not reported in the literature

TABLE 14: THE PAPOVAVIRIDAE IN VITRO

Virus	will infect	will replicate	will transform
Polyoma	+	+	+
SV40	+	+	+
BKV	+	+	+
JCV	+	+	+ / -
K-virus	+	-	+
RKV	+	+ / -	-
HD virus	+	+	+
STMV	+	+	-
HPV	+	+ / -	+
SPV	+	-	+ / -
BPV	+	+ / -	+
Other PV	-	-	-

Note: + = reported in the literature

\- = not reported in the literature

+ / - = unconfirmed report(s) in the literature.

TABLE 15: IMMUNOLOGICAL AND PHYSICOCHEMICAL CHARACTERISTICS OF PAPOVAVIRUSES

Virus	V-antigen	nuclear antigen	cell membrane antigen	H.A.	Diameter (mμ)	Thermo-stability *
Polyoma	+	+	+	+	43-45	+
SV40	+	+	+	-	40-45	+
BKV	+	+	+	+	40.5-44	-
JCV	+	+	+	+	38-43.5	+
K-virus	-	-	-	+	40-50	+
RKV	-	-	-	+	47	+
STMV	-	-	-	-	40-45	-
OMPV	-	-	-	-	33	-
HPV	+	+	+	-	33-60	-
SPV	+	+	+	+ / -	26-60	+
BPV	+	-	+	+	35-54	-
Canine OPV	+	-	+ / -	-	40-50	-
Deer FV	-	-	-	-	50-53	-
ROPV	-	-	-	-	40-52	+
FPV	-	-	-	-	50-53.5	-
Equine PV	-	-	-	-	-	-

\+ - present or positive

\- - not reported or negative

\+ / - - possible or probable

* - virus survives heating at 60°C for 30 minutes

The literature review indicates the great research effort that has been made during the last five years - the next five years may well render a large amount of current speculation as misguided and obsolete.

1.8 General Introduction

Bovine papilloma virus normally causes benign tumours in cattle and is of little economic consequence. The major loss in non-bracken areas is related to the damage to hides and skins. Occasional outbreaks can occur where the effects are severe, especially so in the case of anogenital fibropapillomatosis causing reduced reproductive performance and occasionally death, due to obstruction caused by the tumour or due to secondary infection. Epidemiologically, cattle are, unlike man, isolated within individual herds for the greater period of their lives.

When BPV is endemic in a herd, it is thought that the close contact afforded by winter housing results in transmission to susceptible younger animals. Fibropapillomas on the skin therefore usually appear in Spring on anteroventral skin sites of animals up to 2 years of age (Bagdonas and Olson, 1953). When a herd has been free of BPV for a number of years, the introduction of a new animal may precipitate an outbreak of fibropapillomatosis affecting animals of all ages, sometimes with severe economic consequences. Additionally, normal veterinary procedures such as pregnancy testing and dehorning can lead to severe outbreaks in susceptible herds (Tweddle and White, 1977).

A major cause of economic loss occurs in areas where bracken fern and related species (Pteridium aquilinum, esculentum, Cheilanthes sieberi and tenuifolia) is prevalent (McKenzie, 1978). The interaction between bracken and BPV is unclear but some authors have suggested a co-carcinogenic effect - the prolonged intake of bracken leading to reduced immunocompetence allowing BPV induced lesions of the urinary bladder and upper alimentary tract to proceed to malignancy (Jarrett et al., 1978 and references therein). The absence of reports of BPV-related malignant tumours on the skin or other mucosal surfaces in cattle from bracken areas indicates that other factors may be involved. Such factors include variability in immune surveillance at different sites and the existence of different strains or types of BPV.

BPV is also important as a model for papilloma virus infections of other species, including man. Apart from the unresolved question of whether BPV can itself infect man (see Frenz, 1941; Schultz, 1908; Grenier et al., 1976; Perel and Lumpkin, 1976., Rowson and Mahy, 1967), BPV does have a wider host specificity (Olson et al., 1969) than the other papillomaviruses. It has been related to the development of benign and malignant tumours under natural conditions. The study of BPV may therefore lead to useful techniques and observations applicable to other papillomavirus infections.

This thesis will report the results of investigations into the in vitro and in vivo effects of BPV and BPV transformed cells; the use of BPV in vitro transformation of cells in the development of a quantal assay for BPV and anti-BPV transformed cell antibodies, the results of an abattoir survey coupled with experimental virus transmission and immunological results suggesting the existence of several types of BPV; and the effects of pasteurisation on BPV in vivo and in vitro. Reports of aspects of the work in this thesis will be published elsewhere and the reader is referred to the references listed at the end of this thesis (Meischke, 1978 a - e).

Chapter 2. IN VIVO EFFECTS OF BOVINE PAPILLOMA VIRUS: AN ABATTOIR SURVEY OF BOVINE PAPILLOMATOSIS AND COMPARISON, BY ELECTRON MICROSCOPY AND ELECTROPHORESIS OF BPV ISOLATED FROM VARIOUS LESION TYPES.

2.1 Introduction

Bovine papilloma and fibropapilloma are benign tumours which occur at many different anatomical sites. The common cutaneous fibropapilloma, most frequently found on anterio-ventral skin sites (Frenz, 1941; Bagdonas and Olson, 1953) is a self limiting disease of young cattle. Fibropapillomas of the penis and vaginal mucosa (McEntee, 1951) also occur and may be venereally transmitted in a similar fashion to the human genital wart, condyloma acuminatum (Delap et al., 1976). Papillomas and fibropapillomas of the upper alimentary tract and the teats have been reported earlier (Delap et al., 1976; Martin et al., 1972), and Jarrett et al., (1978a) have shown a 19% prevalence of alimentary papillomas in a large abattoir survey. The demonstration of bovine papilloma virus (BPV) in both tissue extracts and sections (Jarrett et al., 1978a) led to the suggestion that a possible source of virus causing alimentary papillomas was teat papillomatosis (Jarrett et al., 1978b). Frequent observations appear in the literature of upper respiratory and alimentary tract papillomas in children whose mother had been infected with condyloma acuminatum during pregnancy (Wallenborn, 1976). Gissmann et al., (1977) have distinguished four different types of human papilloma virus (HPV) and postulate that further types of HPV could exist in condyloma acuminata and laryngeal papillomas as well as some skin warts.

This part reports the results of a survey of 721 cattle yielding 2,427 papillomas and comparison of various skin and teat virus isolates by the electron microscope and polyacrylamide gel electrophoresis.

2.2 Materials and Methods

2.2.1 Cattle Survey

At approximately weekly intervals, all cattle killed on one day at Glasgow abattoir were examined for the presence of papilloma on all sites of the integument. The site(s) of any papillomas as well as the sex and parity

were recorded for each animal. Every affected teat, scrotum or piece of skin was removed and examined the same day in the laboratory. Skin and teat samples were kept in separate containers. Individual teat papillomas were removed after classification into three macroscopic types and stored separately in PBS/Glycerol held at 4°C. The location, number and classification of each papilloma was documented. A number of papillomas from each macroscopic category were sampled, placed in 10% formol saline and glutaraldehyde fixatives for further histopathologic and electron microscopic (EM) examination.

2.2.2 Virus Extraction

Pooled and single case papillomas were homogenised using a Silverson blender into a 10% W/V crude suspension in phosphate buffered saline (PBS). Purified virus was prepared according to methods described earlier (Lancaster et al., 1976) but without the use of detergent or ultrasound to aid dispersion.

2.2.3 Papilloma Classification

All teat papillomas were categorised into the following types based on gross appearance. A. Frond type - these acuminate lesions had multiple filiform projections of a delicate texture longer than 2mm and with or without a fibromatous base. Papillomas with projections < 2mm were placed into one of the following categories (Figure 2). B. Flat and Round type - fibropapillomas with a fibromatous base, often covered with apparently normal epithelium but sometimes with hyperkeratotic or papillomatous epithelium with filiform projections < 2mm long (Figure 3). C. Rice Grain lesions occurred as focal areas of papillomatous epithelium rarely > 1cm diameter (Figure 4). No macroscopic filiform projections and no fibromatous involvement were visible. In multiple cases these lesions coalesced to form large masses of papillomatous tissue, occasionally covering the whole teat.

2.2.4 Electron Microscopic Examination

All crude suspensions from teat papilloma were negatively stained with phosphotungstic acid (PTA) and examined for the presence of BPV. Purified

Figure 2: Teat papilloma of the Frond type - notice delicate filiform projections > 2mm in length. Bar = 2cm.

Figure 3: Teat papilloma of the Flat and Round type - notice absence of filiform projections > 2mm in length. Histologically (not shown) this lesion was a fibropapilloma. Bar = 2cm.

Figure 4: Teat papillomatosis of the Rice Grain type. These lesions are macroscopically and microscopically similar to focal epithelial hyperplasia in man. Note absence of filiform projections and the discrete epithelial nature of the lesions. Bar = 2cm.

preparations were similarly examined both to confirm the presence of BPV and to subjectively assess the purity of individual preparations. A minimum of 10 electron-micrographs were taken of each preparation for further analysis.

2.2.5 Virus Measurement

Electron micrographs of a standard 3,160 lines/mm grid taken concurrently and at the same indicated magnification as electromicrographs of purified virus preparations were measured under a Leitz surgical microscope. The mean distance between the grid lines of electron-micrographs was divided by the known distance between the grid lines and the specimen to give the actual magnification. Individual particles were measured from a minimum of five different electron micrographs. Two diameter measurements at right angles to each other were made of each virus particle. Only "full" particles were measured. Care was taken to include equal numbers of particles from dark and light backgrounds in an attempt to equalise differential stain penetration.

Purified virus samples from the same case were prepared at different times to detect any time or purification effects on particle diameter.

2.2.6 Polyacrylamide Gel Electrophoresis

The amount of protein in each purified virus sample was determined according to the method of Lowry. Samples for electrophoresis were dissolved in sample buffer (0.0625 M Tris.HCl pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 5% B mercaptoethanol) and boiled for three minutes. The cooled samples were then applied to discontinuous SDS - polyacrylamide slab gels prepared according to the method of Laemmli (1970). Separating gels contained either 8% or 11% acrylamide. Approximately 20 mg of protein was applied to each well. A constant potential of 50V was applied during stacking, and this was increased to 100V for the remainder of the run. Electrophoresis was stopped when the tracker dye was approximately 1cm from the bottom of the gel. Proteins were visualized by staining with Cromassie Blue R.

2.2.7 Papilloma Scraping

Twenty individual papillomas selected at random from each classification group were scraped with a sterile scalpel blade taking care that only epithelial tissue was being removed. Scrapings were placed in 1 ml Tris saline with EDTA and after gentle agitation were subjected to three freeze-thaw cycles. During each cycle the sample was held - 20°C for ten minutes, thawed in a 37°C water bath for ten minutes and gently agitated. After completion of the third cycle the sample was negatively stained with PTA and examined for the presence of BPV. Samples were regarded as negative if BPV could not be seen after fifteen minutes searching of at least two separate preparations. Those papilloma which yielded negative samples were re-sampled on a different site and re-examined under the electron microscope.

2.3 Results

All cattle killed on each of five days were treated as separate sub-surveys. Table 16 summarises the results of the total survey (Appendix A contains the detailed results of the survey). Standard deviations were calculated using the five subsurvey means to test the significance of differences between groups as listed on Table 17 using the Students t-test. 20.8± 5.8% of male cattle; 48.2± 3.7% of parous and 31.0± 7.5% of nulliparous female cattle had teat papillomatosis. The differences between male cattle and female cattle were significant. The average number of teats with papillomas per affected animal was 1.4± 0.3 in male cattle; 2.3± 0.1 in parous and 2.1± 0.3 in nulliparous female cattle. These differences were significant. Throughout the whole survey 36% of cattle had teat papillomatosis, each affected animal had approximately two teats with papillomas on them.

Table 18 shows the proportion of the three papilloma types in a total of 2,427 papillomas found in this survey. Approximately half (52.7± 12.4%) were rice grain lesions (RG), 26.9± 8.2% were of the frond type (F) and 20.4± 5.9% were of the flat and round type (FR). An average of 5.1± 1 papillomas per affected teat is reported.

TABLE 16: Number of Cattle and Teats affected with Papillomatosis

Type of Animal*	M	C	H	MCH
Animals Examined	167	328	226	721
Animals Affected	35	257	68	260
Teats Affected	49	365	141	555
% Animals Affected ± S.D.	20.8± 5.3%	48.2± 3.7%	31.0± 7.5%	36%
Affected Teats/ Affected Animal ± S.D.	1.4± 0.3	2.3± 0.1	2.1± 0.3	2.1±0.2

* M - all male animals - bulls or castrates

C - cows - all parous female animals

H - heifers - all nulliparous female animals.

TABLE 17: t-test of mean differences in % animals affected and number of affected teats per affected animal.

	$M/_C$ +	$C/_H$ +	$H/_M$ +
Difference in % cattle with teat papilloma			
t-value	5.97	1.25	3.99
degrees of freedom	8	8	8
significance	***	n.s.	**
Difference in number affected teats per affected animal			
t-value	9.52	4.61	2.49
degrees of freedom	8	8	8
significance	***	**	*

\+ explained at bottom of Table 1.

*** $p < .001$

** $p < .01$

* $p < .05$

n.s. not significant.

TABLE 18: Proportion of Papilloma Types and Number on Affected Teats

Parameter	Value ± SD
% Frond	26.9± 8.2%
% Flat and round	20.4± 5.9%
% Rice grain	52.7± 12.4%
Number of lesions	2,427
No. of affected teats	472
Number papilloma per affected teat	5.1± 1.0

TABLE 19: Number of Papillomas of each type occurring in three multiplicity groupings from a selected* population.

Multiplicity +	1 - 10	11 - 20	>20	Total
Rice Grain	83	190	164	437
Frond	35	69	85	189
Flat and Round	37	14	0	51

* teats with > 10 papillomas of one type

\+ number of papillomas per teat

The distribution of papillomas according to various multiplicity categories is shown in Figure 1. When papillomas of any type occur in groups of less than ten per teat, the distribution of the F type parallels that of the FR type in both number and shape of curve. When papillomas of any one type occur in groups of > 10, F and FR distribution differ markedly – the F type occurs in greater frequency while the FR type continues to decline. This difference is further illustrated in Table 19. When teats with multiplicities of > 10 papillomas of any one type are examined the F, FR and RG types occur in frequencies comparable with those for the survey as a whole (Table 18) in the 1-10 papilloma per teat group. In the 11 - 20 papilloma per teat group, the FR type is reduced and in the > 20 papilloma per teat group, it has disappeared altogether while the F type has successively increased.

Contrasting with the distribution of the FR + F types, the RG papilloma type shows a different distribution (Figure 1) both in number and shape of curve at multiplicities of < 10 per teat. However, at multiplicities of > 10 (Table 4) the RG type increases in a similar manner to the F type papilloma.

These interactions are further amplified by table 20 which shows the distribution of the three papilloma types and whether they occur as one type or in various combinations. Three theoretical models were tested using prevalence figures listed in table 20. Two models, that the type difference was due to one or three separate virus/host interactions yielded expected distributions at great variance to those observed. The other model, that the type difference was due to two separate virus/host interactions, one comprising F + FR type and the other RG type yielded expected distributions (in brackets on Table 20) not significantly different to those observed. The confounding influences of immunological resistance or susceptibility to the disease led to some differences between the expected and observed distributions of the total and selected population B. Where these influences were absent, close agreement between expected and observed figures occur (population A). (The difference between total affected teat number in Tables 16 and 18 results from the accidental loss of teats from an unselected proportion of cattle slaughtered during part of one day).

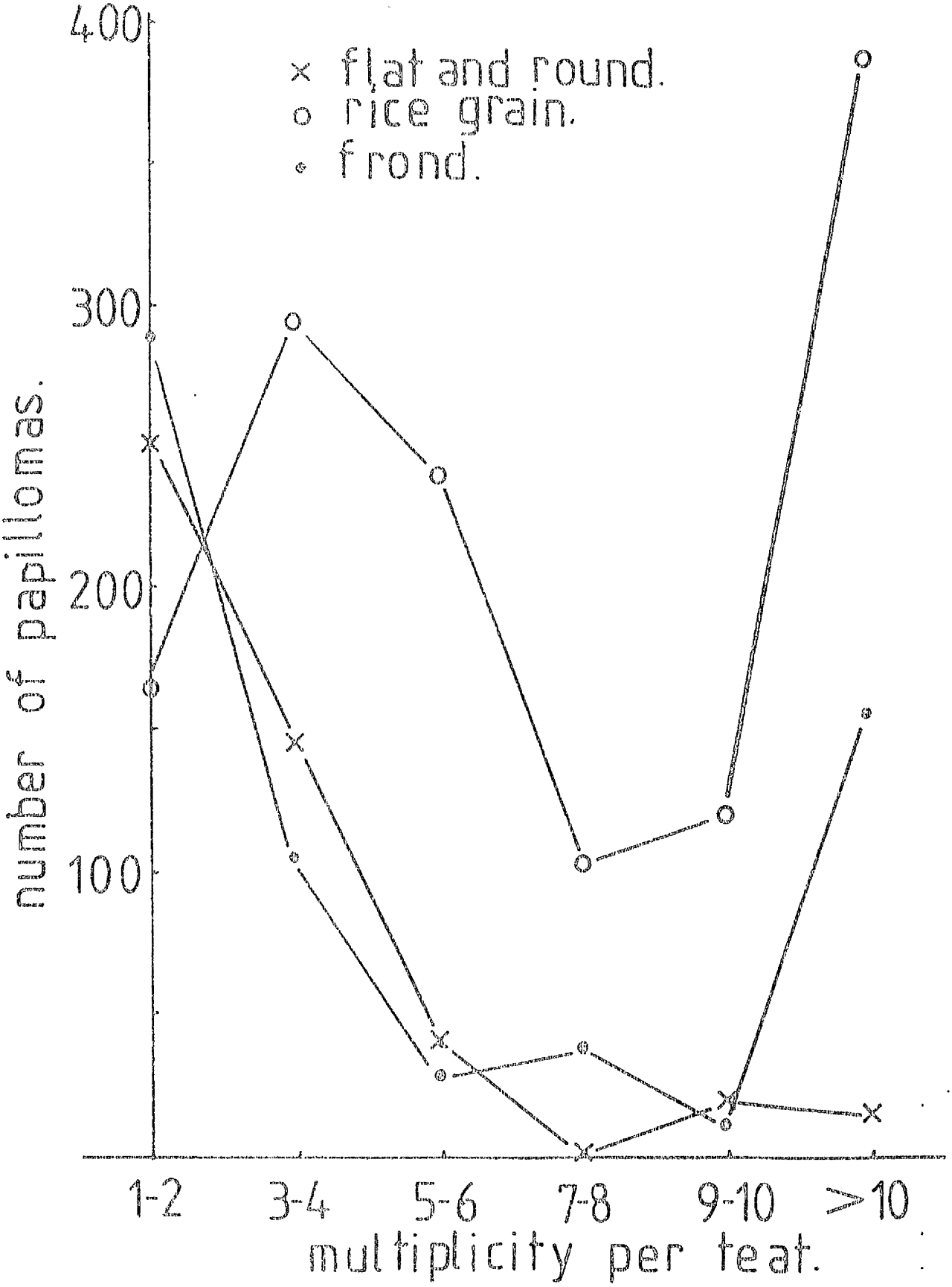


Figure 1: Distribution of papillomas according to multiplicity per teat.

TABLE 20: The distribution of three types of bovine teat papilloma occurring as one type or in various combinations.

Papilloma Type + and Combination	Total Survey				Selected Population*			
	Teats	%	Papillomas	%	Papillomas	%	Papillomas	%
RG	55	50.6	201	23.9 (22.6)	35	8.4 (8.2)	166	29.8 (27.9)
FR	55		112		0		112	
F	106		187		22		165	
FR/F	23		79		0		79	
RG/FR	89	49.4	635	76.1 (77.4)	133	91.6 (91.8)	502	70.2 (72.1)
RG/F	77		588		275		312	
RG/FR/F	67		625		212		413	
Totals	472	100	2427	100	677	100	1,749	

\+ RG - rice grain FR - flat and round F - frond

* selected population A - teats with > 10 papillomas of any one type
B - teats with < 10 papillomas of any type

Note - figures in brackets - expected percentages calculated using the two virus/host interaction hypothesis where F = FR and RG act independently.

Table 21 lists the result of particle measurement of virus from purified preparations of single case and pooled papillomas classed according to their origin. Appendix B contains the detailed particle measurement results. Extracts labelled A are concurrent preparations, as are those labelled B and C. This means that they were collected on the same date, homogenised and purified simultaneously and examined under the electron microscope on the same day. Extracts RGB, RGC, FB, FC and FR are pooled samples, while all the others have single case origins. BPV from RG single case or pooled extract had a mean particle diameter of 47± 3 mμ when RGC is excluded (see Section 3) BPV from TF single case or pooled extracts had a mean diameter of 57± 3 mμ, while BPV from single case extracts of cutaneous bovine papilloma (CBPV) had a mean diameter of 53.5± 4.5 mμ. Virus from a dog papilloma extract had a mean diameter of 65± 2.4 mμ. Using

the students' t-test, differences between these groups were highly significant ($P < .001$), however within group differences were not. Two anomalies occurred and are reported on table 6. Firstly, one sample of a pooled RG preparation (RG C) had a mean particle diameter of 59± 2 mμ, significantly different from ($P<.01$) but similar to the mean diameters of F virus diameters. Secondly the FR virus mean diameter of 53± 4 mμ was not significantly different from that of CBPV extracts. These differences will be discussed further in part 3 following. RG-C and FR produced, on transmission, fibropapillomas. It is likely that pooled samples RGC and FR contained significant contaminant fibropapilloma virus as a result of macroscopic classification errors and particle diameter measurements support this proportion. Figures 5, 6, 7, 8 and 9 show electron-micrographs of skin, Flat and Round, Rice Grain, Frond and Dog Skin papilloma virus extracts.

When twenty individual papillomas from each of the types were scraped and subjected to three freeze/thaw cycles, virus could be seen in negatively stained preparations of all RG and F types examined. However, only 13/20 of the FR types yielded visible virus with a further two of the negative FR papillomas yielding visible virus after a repeat scraping around the junction of normal and papillomatous epithelium. The remaining five FR lesions did not yield visible BPV.

The protein compositions of various purified papilloma virus preparations are shown in figures 10 and 11. The apparent molecular weight (M_r) of the major protein band, which would appear to correspond to VP2 of human papilloma virus (Gissman et al., 1976), varied between isolates. This band seemed to show a consistently higher molecular weight in teat BPV isolates than in cutaneous BPV. The comparative mean molecular weights would be 63,000 and 56,000 respectively. Five teat BPV isolates and one cutaneous BPV isolate are compared in figures 10 and 11. This has been extended to another two teat BPV isolates and three more cutaneous BPV isolates (data not shown) with the same conclusion. Figure 10 also showed quantitative differences in low molecular weight species which had similar M_r values in all BPV isolates. The cutaneous BPV preparation showed much less of these components than the other virus preparations. The canine

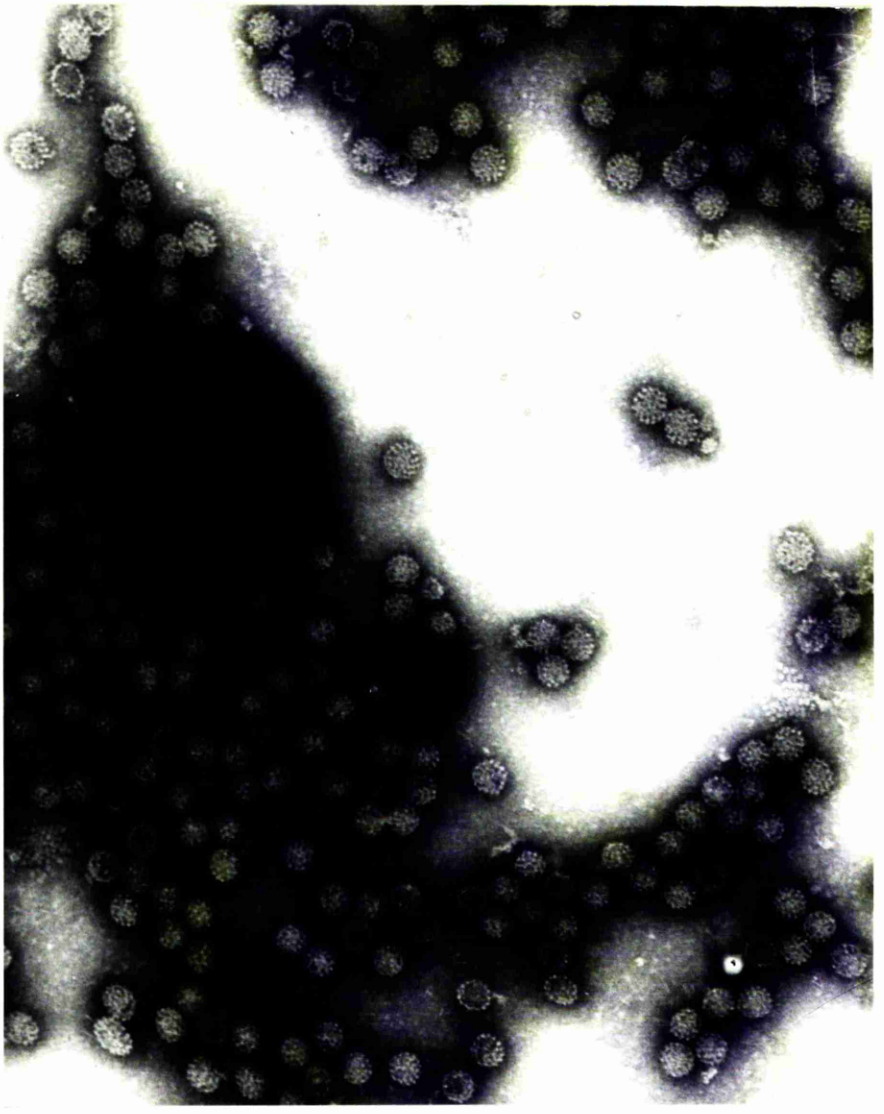


Figure 5: Electron micrograph of negatively stained preparation of bovine skin fibropapilloma virus (CBPV1A). X85,760 ± 1322.

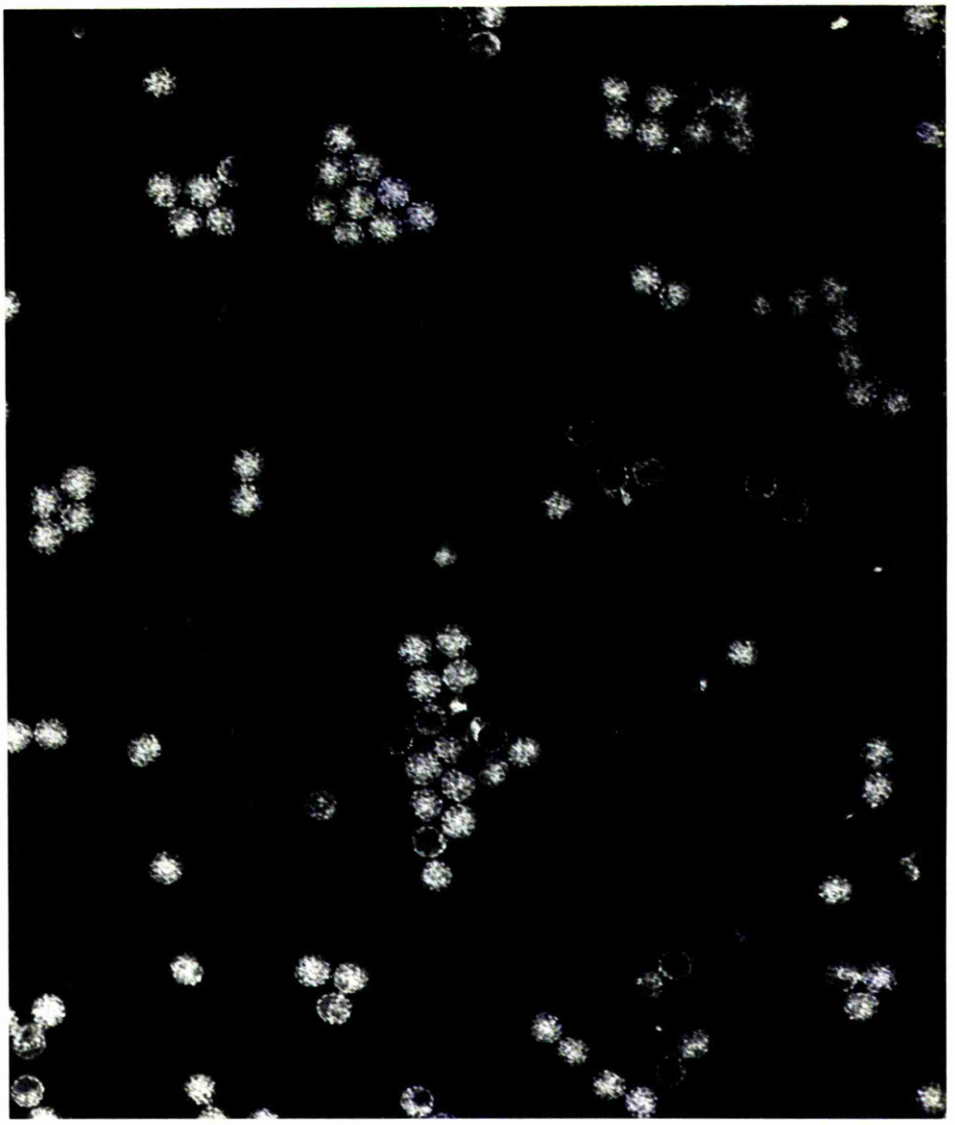


Figure 6: Electron micrograph of negatively stained preparation of bovine teat fibropapilloma virus (FR). x 85760 ± 1322.

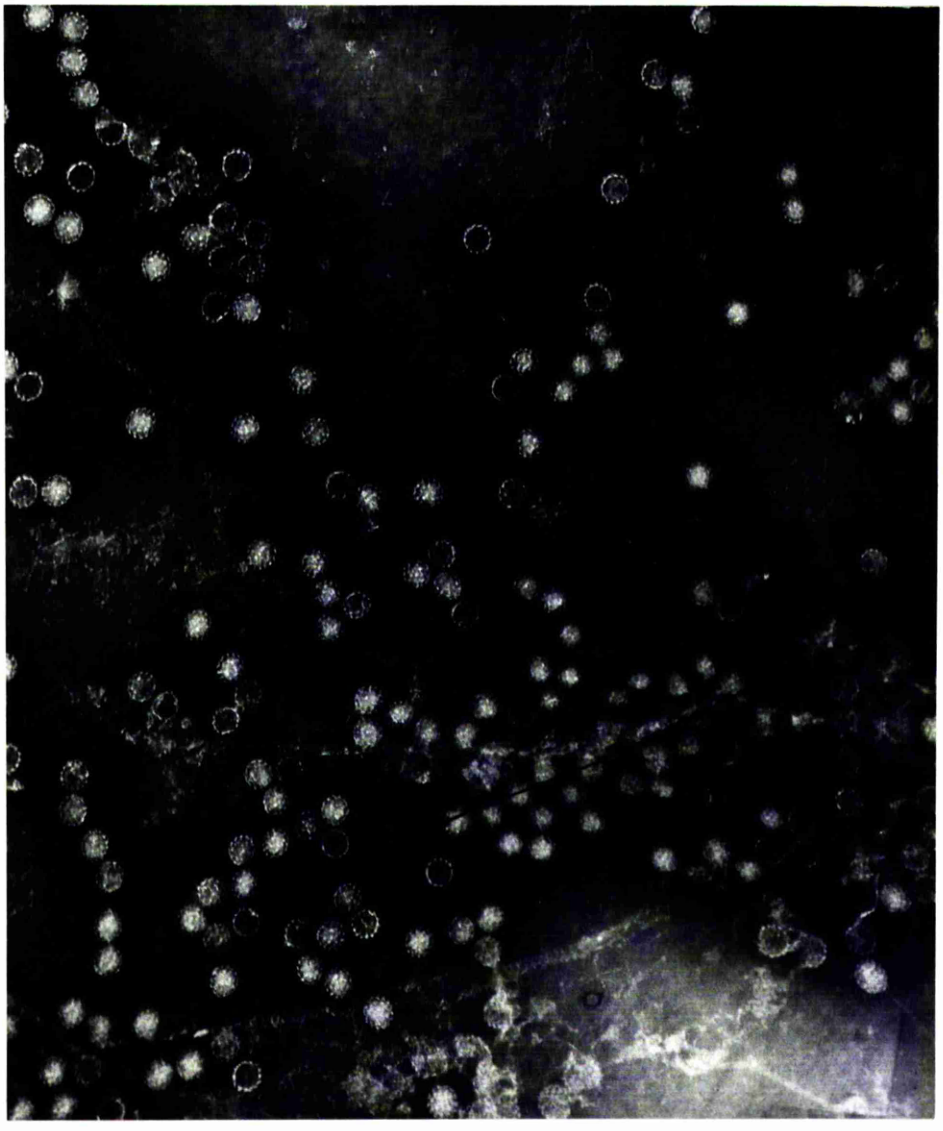


Figure 7: Electron micrograph of negatively stained preparation of bovine teat rice grain papilloma virus (RGA). x 85760 ± 1322.

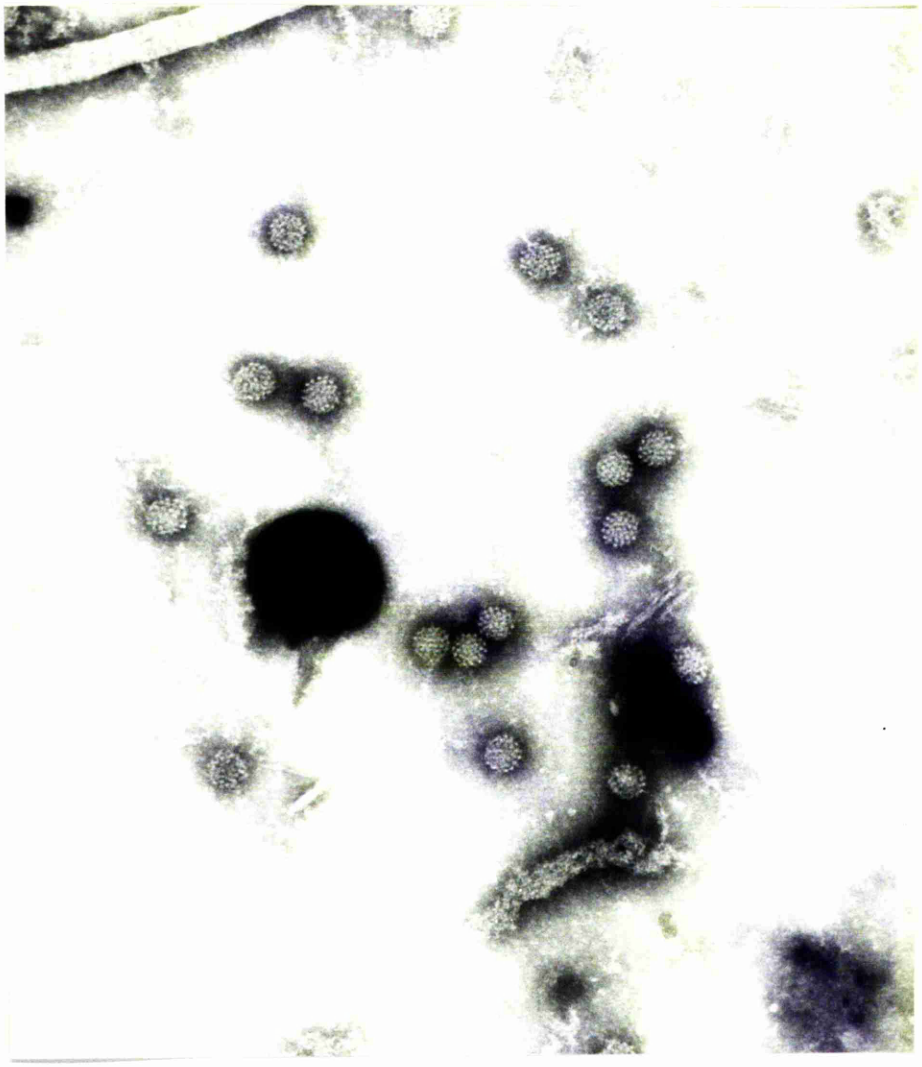


Figure 8: Electron micrograph of negatively stained preparation of bovine teat papilloma virus (FA). x 85760 ± 1322.

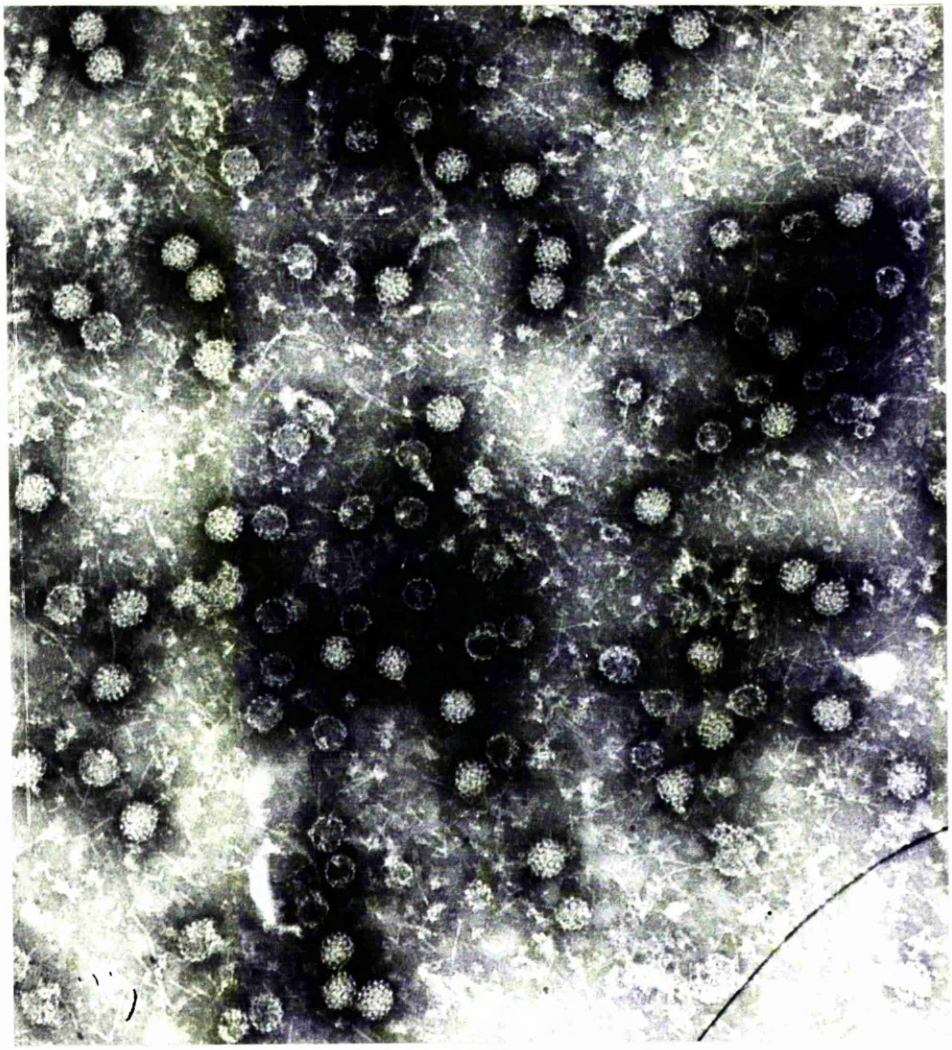


Figure 9: Electron micrograph of negatively stained preparation of dog skin papilloma virus (DP). x 85760 ± 1322.

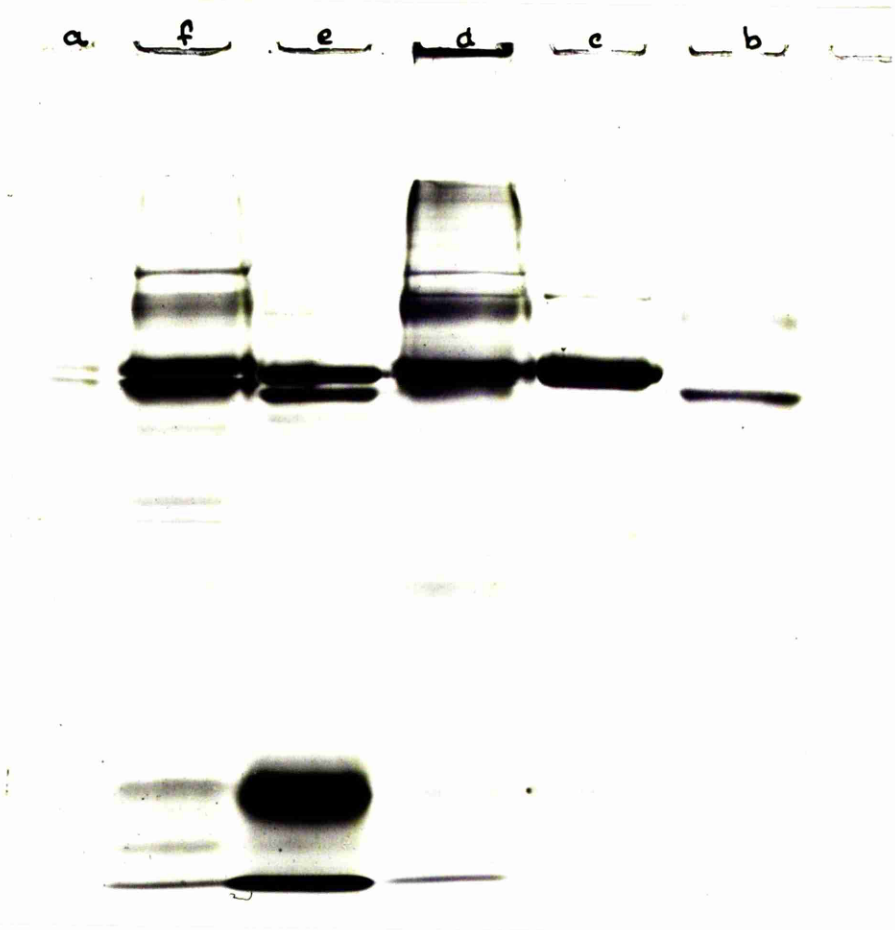


Figure 10: SDS - polyacrylamide gel electrophoresis of bovine papilloma extracts from different lesion types. Samples were separated on an 11% acrylamide gel. Lane a) standard marker proteins (phosphorylase-a, 94000 M_r; catalase, 60000 M_r; alcohol dehydrogenase, 41000 M_r; carbonic anhydrase, 29000 M_r; cytochrome-c, 11700 M_r) Lane b) C BPVIA; lane c) FR; lane d) FA; lane e) DP; lane f) RGA.

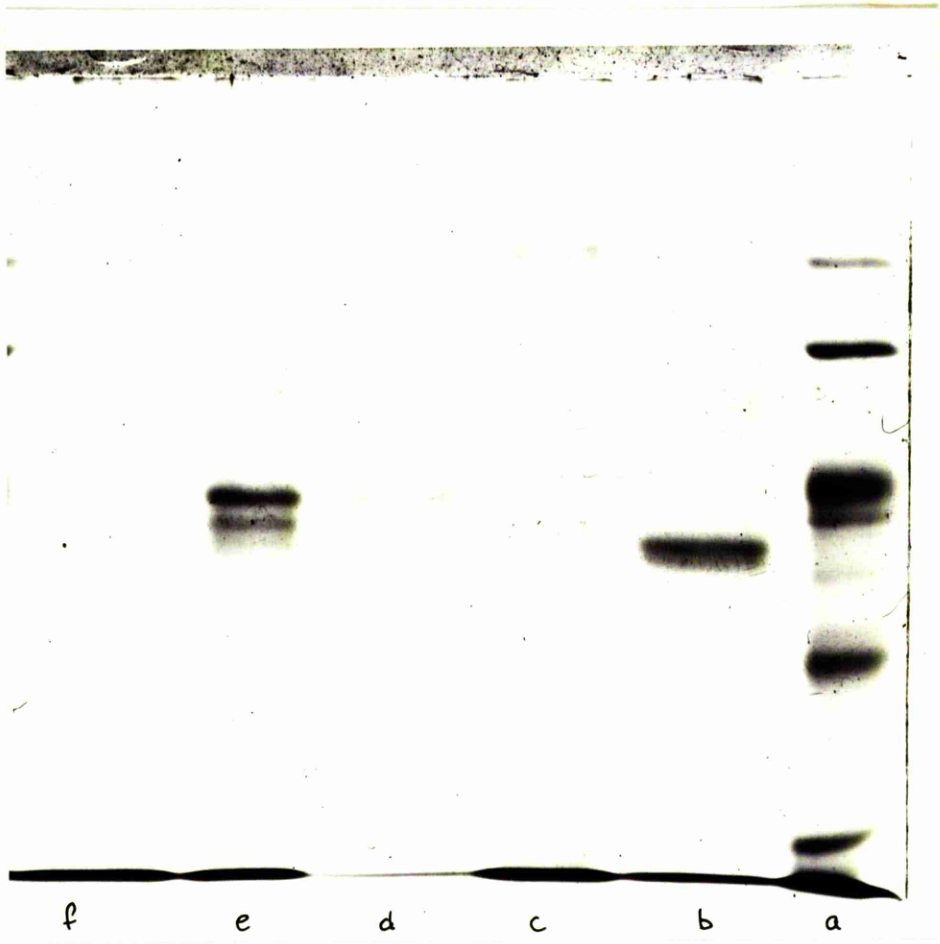


Figure 11: SDS - polyacrylamide gel electrophoresis of bovine papilloma extracts from different lesion types. Samples were separated on an 8% acrylamide gel. a) standard proteins (B- galactose,130000M[illegible].; rest as for figure 10 with the exception of cytochrome-c); b)CBPV1B; c)CBPV1B; d) FB; e)RGB; f)CBPV2.

TABLE 21: Mean BPV particle diameter measured on electronmicrographs[o] of negatively stained purified extracts of various papilloma samples.

Extract of	Mean Diameter ± SD (mμ)	Number Measured
* RG A	47.6 ± 2.9	25
RG B	46.7 ± 3.5	200
RG C	58.7 ± 2.2	100
F A	57.6 ± 2.6	50
F B	56.5 ± 3.3	125
F C	56.9 ± 3.4	75
FR	53.0 ± 4.1	175
+CBPV1 A	51.7 ± 4.8	25
CBPV1 B	53.6 ± 4.5	75
CBPV2	54.9 ± 3.6	150
x DP	65.1 ± 2.4	50

\+ cutaneous bovine papilloma virus extract

X dog papilloma extract

* explained at foot of table 20.

o actual magnification x 8[illegible]760 ± 1322.

papilloma virus showed a distinctive pattern, with two bands in the "VP 2" region which did not comigrate with any band in the BPV preparations.

There was also a very prominent band at 15,000 M_r. Some preparations (Fig. 11, lanes b and c) showed an excess of high molecular weight bands over any band in the expected "VP 2" region. Since the purification procedure involved only a single CSCl gradient step, it seems likely that these bands represent tissue protein contamination. Although these preparations contained virus when examined by electron microscopy, larger quantities of tissue had been extracted increasing the likelihood of seeing minor contaminant species in SDS.

2.4 Discussion

Normally bovine cutaneous fibropapilloma affect cattle less than two years of age, has a well recognised viral aetiology and is a self limiting disease. (Frenz, 1941; Bagdonas and Olson, 1953). The results of a recent survey suggest that bovine alimentary papillomatosis presents a different epidemiological picture with an overall prevalence of 19%. No significant differences were found between cattle aged < 3 years and cattle aged > 3 years in either papilloma prevalence or multiplicity. Virus-like particles were found in four of seven papillomas examined as thin sections under the EM while light microscopy of 100 cases revealed a 13% prevalence of type A intranuclear inclusion bodies (Jarrett et al., 1978a).

This part reports that teat papillomatosis presents an epidemiological situation markedly different to that reported previously for either cutaneous or alimentary papillomatosis. With the exception of one cow, all papilloma observed were limited to the hairless portion of the teats with random distribution over that limited area. The one exception was a cow showing a mixed type population of 155 teat papilloma on the five teats she possessed - two of these papilloma were 3 cm above the hair-line, the rest were restricted to the hairless portion of the teats. Concurrent examination of the rest of the integument of each of the 721 cattle yielded two animals with cutaneous fibro-papillomatosis and four with scrotal papillomas, a respective prevalence of 0.3% and 2.4%. While papillomas were never observed within the teat canal, they frequently formed clusters around the teat orifice,

making it quite conceivable that milk from these animals would contain virus released by the superficial trauma of suckling calves or machine milking. The effect of pasteurisation on infectivity of BPV extracted from the three types of lesions will be reported in part 6 of this thesis.

While there were no significant differences between heifers (nulliparous females) and cows (parous females) in the proportional number of animals affected, there was a significant increase in the number of affected teats per animal. Male animals showed a significantly lower prevalence of teat papillomatosis with fewer teats affected per animal. The male-female differences may simply be a reflection of relative differences in the size of teats, but hormonal or behavioural differences cannot be disregarded. The cow-heifer differences indicate a higher prevalence with increasing age or parity,but also that teat papillomatosis is widespread in the population before first calving.

Separation of the three papilloma types on macroscopic appearance proved relatively easy. There were cases where individual lesions were classed as FR when later detailed examination proved them to be either F or RG, but, relative to the total numbers involved, the system worked well.

The unlikely finding that the total number of papillomas comprised approximately half (52.7%)RG types and half F + FR (26.9% and 20.4% respectively) coupled with the fact that F and FR both possessed the common factor of connective tissue involvement, led to the first suspicion that teat papillomatosis was a mixture of two closely related but separable disease processes. This suspicion was enhanced by the finding (fig. 1) that F and FR had similar distributions at lower multiplicities (<10) while RG had a distribution different in both number and shape of curve. The distributions at high (>10) multiplicities, presented a different picture where RG and F increased rapidly while FR continued its' decline (table 19). This latter feature suggested that FR is a regressing form of F, explaining the earlier observation that, overall, F exceeds FR in number (26.9% compared with 20.4%).

When three hypotheses were put, that these type differences were due to 1, 2 or 3 separate virus/host interactions, the only hypothesis which yielded comparable expected and observed proportions in lesion distribution

(table 20) was that two virus/host interactions were occurring. If one, F and FR and the other, RG are treated as separate, independent processes then they would be expected to occur together in 77.4% of the total papilloma population, 72.1% of the selected population B (<10) and 91.8% of the selected population A (> 10). Conversely, they would occur alone in 22.4% of the total, 29.9% of selected B and 8.2% of selected A papilloma populations. The respective observed percentages are 23.9%, 29.8% and 8.4% and these are not significantly different from the expected calculated using the two factor hypothesis. Observed and expected populations were at great variance when a single or three factor hypothesis were used. Agreement between expected and observed figures is best in selected population A with only a difference of 0.2%. High multiplicity population A also contains a low FR complement so it is likely that this population is immunologically less competent to deal with the disease. In the total survey and population B, the confounding influences of individual immunity and susceptibility must lead to greater variance between expected and observed frequencies.

The demonstration of papilloma virus in scrapings from all three types represents a new finding. The technique used has not been previously reported and may have application in clinical medicine both because it allows sampling of individual papillomas and because it yields an answer within an hour of original sampling.

Further support for the suggestion that FR is a regressing form comes from the finding that virus was more difficult to obtain by scrape sampling (in 25%, virus could not be demonstrated).

Measurement of BPV particle diameters on electron micrographs of negatively stained preparations of CsCl purified extracts proved difficult and time consuming. All F samples showed a significantly higher mean diameter than CBPV samples while two of the three RG samples were significantly lower. The mean particle diameters reported in this paper are on average 5 mμ higher than those reported previously - this may be due to the fact that negatively stained preparations were used or that our technique is different to that used in other laboratories.

The cutaneous and teat BPV preparations which have been examined showed distinct polypeptide compositions. This difference seems to be reproducible. To date seven teat BPV and four cutaneous BPV isolates have been examined. G issman et al., (1976), showed that one human papilloma virus (HPV) isolate, HPV 4, was unrelated to isolates HPV 1-3, and showed a different polypeptide distribution. They were also unrelated by DNA endonuclease cleavage pattern, CRNA hybridisation and serological specificity. No such criteria are yet available for BPV isolates, but it will be informative to compare them in this manner. Some characterisation of HPV proteins has been reported by Pfister et al., (1977) who found VP2, 3 and 4 to be highly related by examining BrCN cleavage patterns. They were, however, unable to establish that VP3 was a proteolytic cleavage product of VP 2 by this technique, and suggested that the M_r differences may be due to acetylation, deamination or glycosylation. This is of particular interest with regard to the teat "rice-grain" virus which generally showed a second molecular species, equimolar with "VP 2" and of slightly lower molecular weight.

Quantitative differences in low molecular weight species (12 to 15,000 M_r) were also found. Cutaneous BPV showed very little of these components. A problem which was encountered with some virus preparations was the presence of what appear to be contaminant tissue proteins. The virus purification protocol which was used was as brief as possible, since it was thought important to leave the virus structure intact.

2.5 Summary

In a survey of 721 cattle passing through Glasgow abattoir, 36% had teat papillomatosis. Affected animals possessed an average of five papillomas on each of two affected teats. Significant sex differences occurred in the prevalence of the disease, and nulliparous female cattle had significantly fewer teats affected than parous females.

When individual papilloma were divided according to macroscopic appearance, 27% were of the frond(F) type; 20% were of the flat and round (FR) type and 53% were rice grain (RG) lesions. The F and FR types had the common feature of connective tissue involvement and showed similar

distributions in the population examined. The RG type had a significantly different distribution and lacked connective tissue involvement. The observed and expected frequencies of association between the three types were not different only when a model assuming two separate, independent virus/host interactions was used. In this model F and FR types formed one and RG the other virus/host interaction. From higher multiplicity cases, it was concluded that FR could be a regressing form of the F type.

Bovine Papilloma Virus (BPV) could be demonstrated in all F and RG types examined, but only in 75% of FR type papillomas. Mean particle diameters of RG, F_1 cutaneous fibropapilloma and canine skin papilloma viruses were significantly different from each other.

Separate purified viral extracts from 4 cutaneous and 7 teat papilloma samples were compared by polyacrylamide gel electrophoresis. All purified teat virus samples showed a major capsid protein band which had a consistently higher apparent molecular weight than comparable cutaneous virus extracts.

This part suggests that two different BPV virus/host interactions occur in bovine teat papillomatosis. Virus extracted from teat papilloma was significantly different from the virus of cutaneous fibropapillomatosis in both particle diameter and apparent molecular weight of major viral capsid protein.

CHAPTER 3

IN VIVO EFFECTS OF BOVINE PAPILLOMA VIRUS: EXPERIMENTAL TRANSMISSION TO CATTLE OF BOVINE PAPILLOMA VIRUS (BPV) EXTRACTED FROM MORPHOLOGICALLY SEPARABLE TEAT AND CUTANEOUS LESIONS AND THE EFFECTS OF INOCULATION OF BPV TRANSFORMED FOETAL BOVINE CELLS.

3.1 Introduction

Bovine papilloma virus (BPV) has been extracted from a variety of lesions occurring on different anatomical sites in cattle. BPV was found to be the causative agent of cutaneous fibropapillomas by Creech (1929), genital fibropapillomas (McEntee, 1952), and it may be associated with chronic enzootic haematuria of cattle (Olson et al., 1965). Atypical cutaneous papillomas have been shown to contain BPV (Barthold et al., 1974). BPV has been extracted from alimentary papillomas (Jarrett et al., 1978); teat papillomas, teat fibropapillomas and teat rice grain lesions, similar to focal epithelial hyperplasia in man (Chapter 2). In vitro BPV transforms cultures of foetal bovine skin, conjunctiva, meninges and palate (Chapter 7). This chapter reports the results of experimental inoculation of calves with different BPV extracts, BPV transformed foetal bovine cells, and subsequent challenge with the same BPV extracts.

3.2 Materials and Methods

3.2.1 Lesions

BPV extracts were derived from five cattle, each affected with only one of the following morphologically separable lesion types.

A. Rice grain lesions of the bovine teat (Chapter 2), histologically similar to focal epithelial hyperplasia in man (Praetorius-Clausen, 1972).

B. Papilloma without fibroma, previously described as atypical warts on bovine skin (Barthold et al., 1974) and also reported on the bovine teat (Chapter 2).

C. Fibropapilloma occurring on the bovine teat, previously described as flat and round lesions (Chapter 2).

D. Typical fibropapilloma as described in the literature occurring on anterioventral skin sites (Bagdonas and Olson, 1953).

E. Fibropapilloma, presenting as cauliflower like lesions affecting the ano-genital regions and the ventral abdominal wall (McEntee, 1952; Tweddle and White, 1977).

Lesions were removed and stored separately in PBS/glycerol at 4°C. One lesion from each of the five cases was sampled and placed in 10% formol saline for further histopathological examination.

3.2.2 Virus Extraction

After histological confirmation of lesion type, the stored samples were homogenised using a Silverson blender into a 10% w/v crude suspension in phosphate buffered saline (PBS). This suspension was clarified using an SW27 rotor at 10,000 r.p.m. for 20 minutes in a Beckman L-250 ultracentrifuge. The resulting supernatant was then labelled and stored at -70°C until further use. Suspensions were negatively stained with phosphotungstic acid (PTA) and examined under the electron microscope (EM) to confirm the presence of BPV.

3.2.3 Foetal Bovine Cells

Transformation sensitive foetal bovine meningeal

cells (FBMC) and foetal bovine skin cells (FBSC) were prepared as elsewhere described (Chapter 7). A transformed culture was prepared by infecting FBSC and FBMC with a 10^{-3} dilution of a 10% w/v BPV extract of lesions classed as D above. Parallel untransformed and transformed FBSC and FBMC were passaged at weekly intervals using Basal Eagles Medium (BME) with 10% added foetal bovine serum (FBS). Cultures from passages 10 - 12 were used for animal inoculation. Used culture medium was collected, precipitated at $4^{o}C$ with 50% added saturated ammonium sulphate, resuspended in PBS and subjected to differential centrifugation before CsCl gradient purification and EM examination for the presence of BPV. Transformed and control cultures were grown on 5 cm plastic petri dishes for three days, the medium was removed and cells were fixed in situ using glutaraldehyde. After fixation, the cells were removed using a rubber policeman and further processed for thin section E.M.

3.2.4 Animal Inoculation

Ten calves 2 - 4 months old were randomly divided into three groups. (1) Five calves were inoculated with virus isolates from lesions A - E. (2) Three calves were inoculated with transformed cell cultures. (3) Two calves were inoculated with control cell cultures. Two calves, one inoculated with lesion D virus and one inoculated with lesion D BPV transformed cells (FBSC) were kept isolated from the other calves and from each other. The other calves were kept separately in two groups of four - the virus inoculated and the cell inoculated - except during the contact section of the experiment. Virus inoculated calves were given 10% w/v suspensions of BPV from lesions A - E both as 10 intradermal injections and by scarification on both sides of the torso, each calf receiving

virus from only one lesion type. Cell inoculated calves received ten intradermal injections over the lumbar area of the torso as well as three sublingually. Each injection contained 10^6 cells in 0.2 ml PBS, or 0.2 ml of the 10% w/v BPV suspension. All inocula contained 10 mg/ml of sterilised, filtered, activated charcoal to act as a marker for biopsy sampling.

3.2.5 Biopsy Samples

Animals were sampled at fortnightly intervals at both the intradermal and scarification sites throughout the period of the experiment. Each biopsy sample was divided into two, one part was fixed in 10% formol saline, the other was frozen at -70°C for future use. Lesions were scrape-sampled as described earlier (Chapter 2) and examined under the EM to determine the presence of BPV. As indicated on Table 22, lesions from the scarification sites were harvested at intervals and stored in 50% PBS/Glycerol for virus extraction. Blood was collected at fortnightly intervals for haematological examination and serum was stored at -20°C until required for further use (see Chapter 8).

3.2.6 Challenge Experiment

Animals were challenged by two methods. The calves (excluding the two isolated calves) which received virus and showed visible lesions were placed in contact with the cell inoculated calves for a period of eight weeks. All animals were examined at weekly intervals and the presence and type of any lesions developing at non-inoculation sites were noted. The second method involved the challenge of every calf with virus from each of the five lesion types. Scarification sites were prepared by clipping the hair from five 10 x 20 cm major skin areas leaving at least 10 cm of unclipped hair covered skin between adjacent areas.

Challenge sites were observed at weekly intervals and the presence and type of any lesion was noted.

3.2.7 Post Mortem Examination

All calves were subjected to a detailed post mortem examination twelve weeks after challenge by scarification. All systems were examined macroscopically in detail, with special attention given to epithelial tissues involving the alimentary, respiratory and genito-urinary tracts. Lesions found were fixed in 10% formol saline and subsequently processed for histopathological examination.

3.2.8 Histopathology

All samples were routinely stained with haematoxylin and eosin, appropriate special stains were used when necessary.

3.3 Results

3.3.1 Histopathology

A) Figure 12 shows the histological appearance of both the lesions from which BPV was extracted and those produced by transmission to calf 68882 (table 22). Macroscopically, the rice grain lesions are white or flesh coloured, small, elevated nodules, less than 1 cm diameter without papillomatous fronds (acuminate, filiform projections of epithelium). In severe cases, lesions may coalesce to cover large areas. Microscopically, the lesions are characterised by a focal epithelial hyperplasia with marked acanthosis but without parakeratosis. There is little or no hyperkeratosis except in areas between connective tissue papillae. An increased cellular density of the epithelium with mainly horizontal thickening of the epithelial rete pegs, renders the

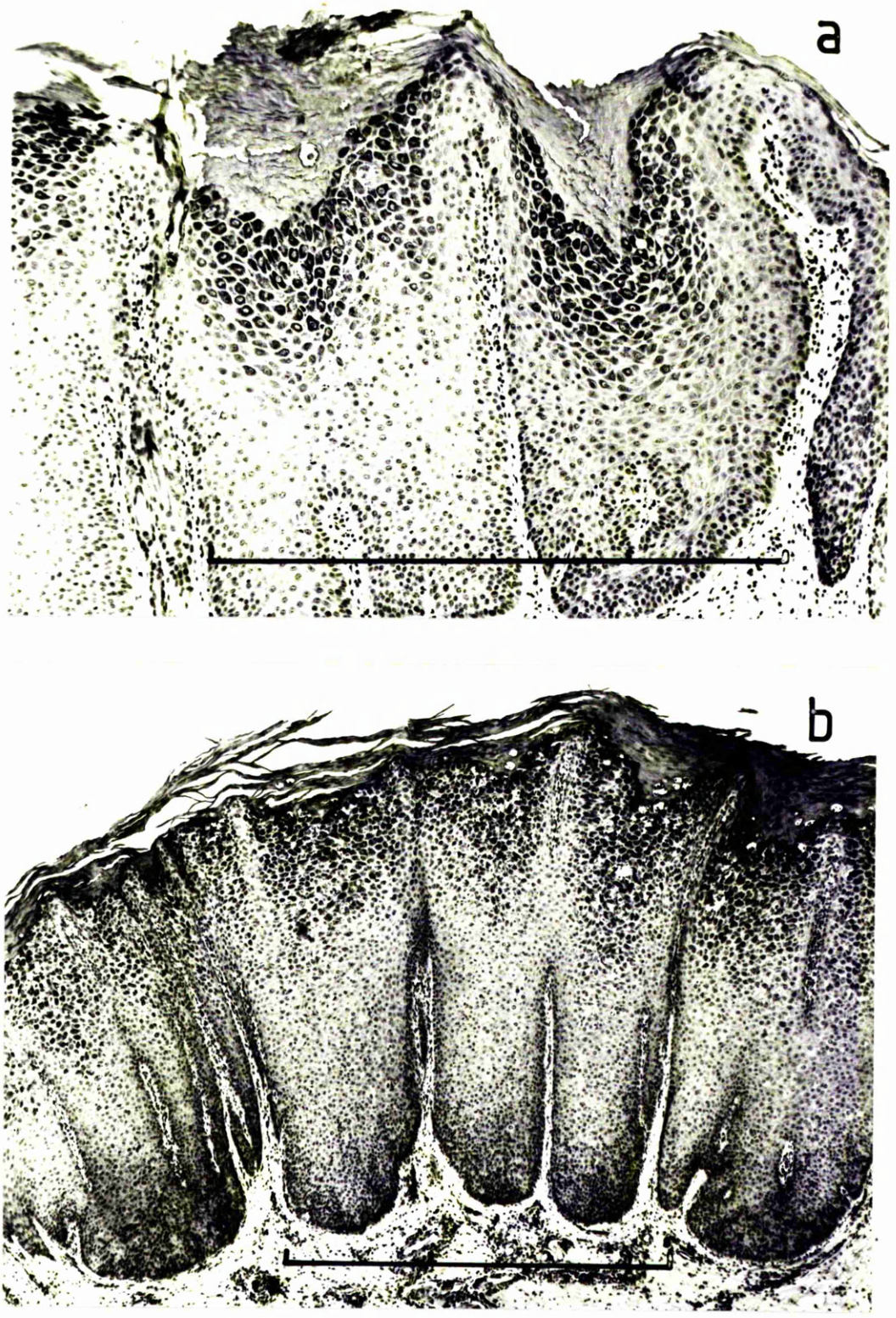


FIGURE 12 - (a) Histological appearance of teat rice grain lesion from which BPV was extracted. (b) Transmitted rice grain lesion on skin of calf 68882. These lesions are similar to focal epithelial hyperplasia in man, and are neither papillomas nor fibropapillomas. Stain H & E Bar = 1mm.

TABLE 22 THE DEVELOPMENT ⊗ AND REGRESSION ° OF LESIONS PRODUCED AT BPV INOCULATION SITES.

CALF	68882	68886	68888	68891	68893
BPV SOURCE *	A	E	B	D	C
TIME POST INOCULATION (weeks)					
2	+	+	+	+	+
4	~~II~~	+	+	+	~~II~~
6	~~III~~	~~III~~ o	~~II~~	~~II~~	~~II~~
8	~~III~~	~~IIII~~ ooo	~~III~~	~~III~~ o	~~III~~
10	~~III~~	~~III~~ oo	~~IIII~~	~~IIII~~ ooo	~~IIII~~
12	~~III~~	~~II~~ o	~~IIII~~	~~III~~ oo	~~IIII~~
14	~~III~~	+ o	IIII	~~II~~ o	~~IIII~~ o
16	~~III~~		~~IIII~~	+ o	~~IIII~~ oo
18	~~III~~		IIII		~~IIII~~ oo
20	~~III~~		IIII		~~IIII~~ o
22	+		~~IIII~~		~~IIII~~
24	+		~~IIII~~		~~IIII~~
LESION TYPE	A	E	B	D	C

o : regression of lesions o = small areas of lymphocyte accumulation oo = large masses of lymphocytes accumulating. ooo = large masses of lymphocyte accumulation with necrosis of involved tissues.

* : lesion types A - E described in Materials & Methods section.

⊗ : development of lesions + visible scarification lines ~~II~~ thickened scarification lines ~~III~~ lesions <1cm diameter ~~IIII~~ lesions >1cm diameter.

connective tissue papillae extremely thin, containing only blood and lymph vessels with almost no connective tissue stroma. The stratum granulosum is markedly thickened with cells showing angular basophilic intracytoplasmic inclusions. In the upper stratum spinosum, occasional basophilic intranuclear inclusions are seen. There is no dermal fibroma and the dermo-epidermal junction is well defined in early lesions. On transmission, lesions were first seen 4 weeks post inoculation and persisted till week 20 (table 22). Regression of the lesions was accompanied by a chronic inflammatory infiltrate surrounding areas of a disrupted epidermo-dermal function, ultimately leading to sloughing of affected epithelium with replacement by plug of fibrin and inflammatory cells. Regression was not accompanied by a massive lymphocyte accumulation as seen with fibropapillomas. Presence of rice grain lesions did not prevent the transmission of fibropapillomas on calf 68882 (table 23). The regression of fibropapillomas (described later) was not accompanied by the regression of rice grain lesions.

B) Figure 13 shows the histological appearance of both the lesions from which BPV was extracted and those produced by transmission to calf 68888 (table 22). Macroscopically, papillomas are circumscribed, exophytic growths with a papillomatous surface (frond length $>$ 2mm) which may be sessile or pedunculated. Microscopically, they are characterised by a papillomatous surface with conspicuous hyperkeratinisation. Predominantly hyperorthokeratinisation but with occasional areas of parakeratosis. The stratum granulosum is

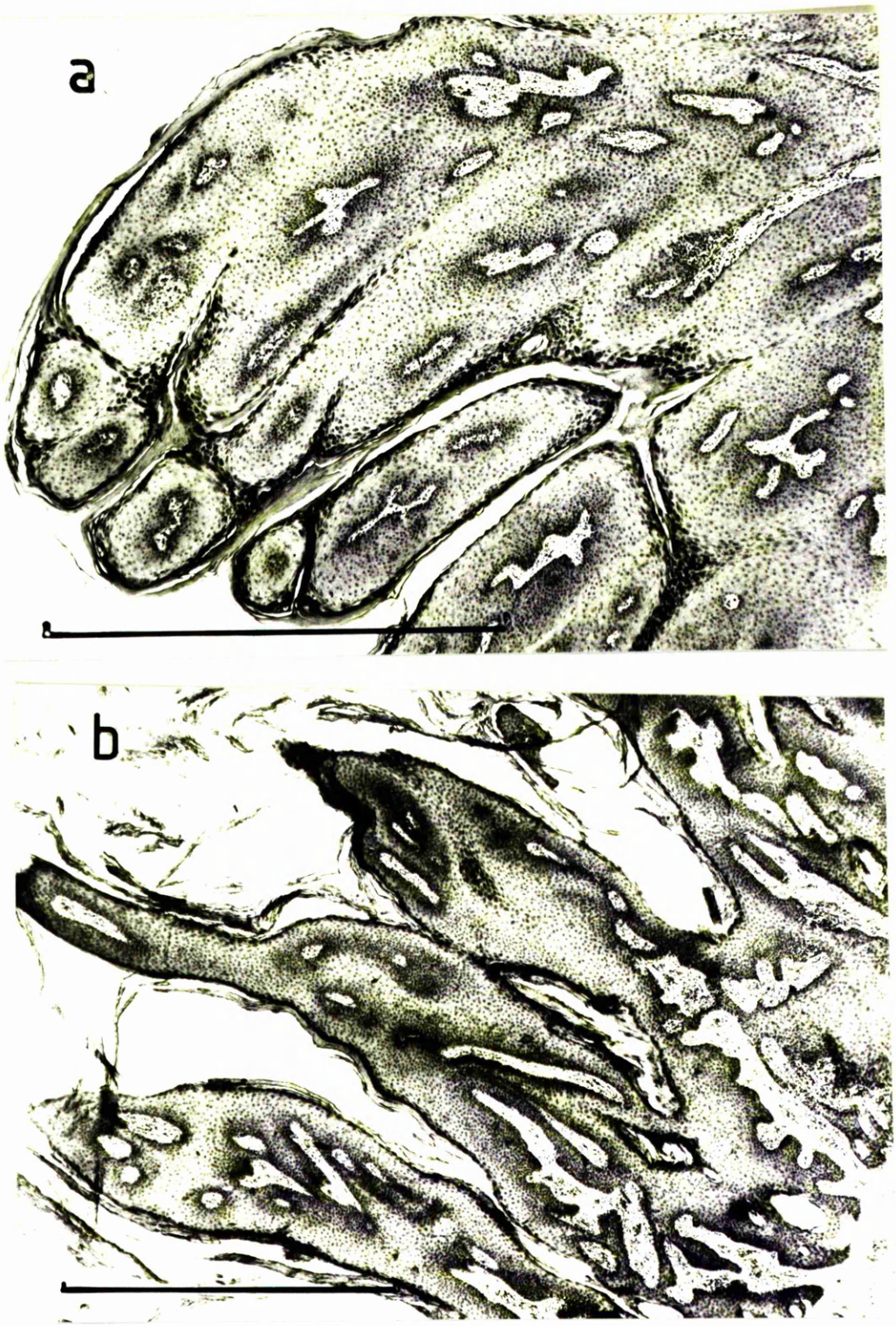


FIGURE 13 - (a) Histological appearance of teat papilloma without fibroma from which BPV was extracted (b) transmitted lesion showing papilloma without fibroma component on skin of calf 68888. These lesions are similar to atypical warts previously reported on bovine skin.
Stain H & E. Bar = 1mm.

markedly thickened, especially in the areas between the papillomatous elevations which form around thin, elongated connective tissue papillae. Epithelial rete bridges often show a characteristic inward bending at the lesion margins. The stratum granulosum contains dense, basophilic intracytoplasmic inclusions, with cells containing intranuclear inclusions in later lesions. The dermo-epidermal junction is well defined in early lesions and there is no accompanying dermal fibroma. On transmission, lesions were first noticed at 8 weeks post inoculation and persisted until post mortem at week 25. In biopsy samples from week 14 till week 25, a chronic inflammatory reaction could be seen with the occasional interruption of affected epidermo-dermal junctions. Large accumulations of lymphocytes, accompanying regression of fibro-papillomas (see later) were not seen. The presence of papillomas did not prevent the development of fibropapillomas on the same calf (table 23) nor was the regression of those fibropapillomas accompanied by the regression of papillomas.

C) Figure 14 shows the histological appearance of both the lesions from which BPV was extracted and those produced by transmission to calf 68893 (table 22). Macroscopically, the lesions present as fibropapill-omas on the bovine teat. They vary widely in size from < 1cm to > 5 cm, occasionally exceeding the size of the teat. They may be pedunculated but more commonly are sessile. The lesions commonly have fronds > 2 mm in length but occasionally these may be absent from part or the whole surface leaving a smooth covering of relatively normal epithelium. In contrast to the lesions reported under E/ following,

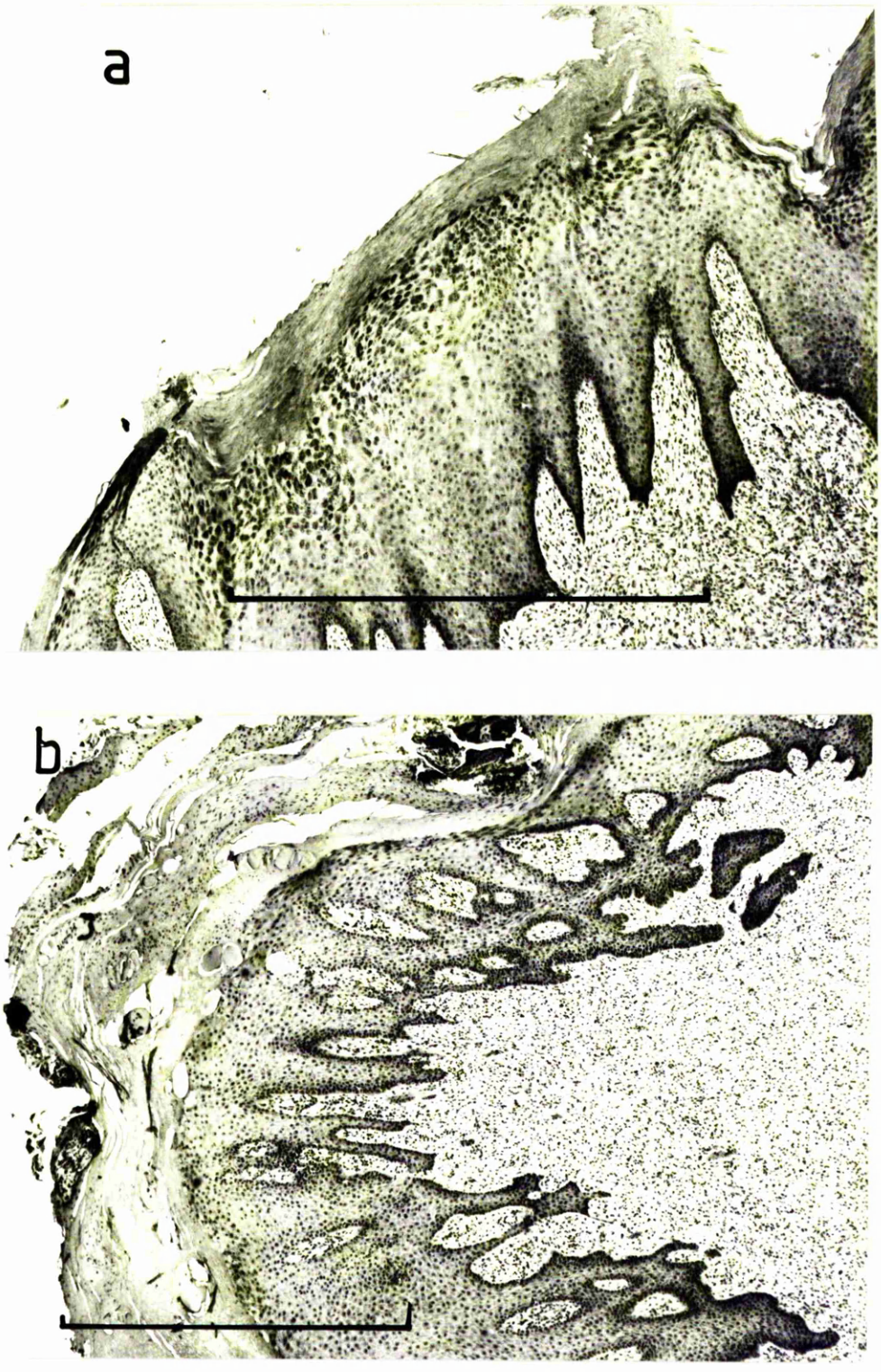


FIGURE 14 - (a) Histological appearance of teat fibropapilloma from which BPV was extracted (b) Transmitted lesion showing fibropapilloma on skin of calf 68893. These lesions are not histologically separable from those illustrated in figure 15.
Stain H & E. Bar = 1mm.

TABLE 23 - CHALLENGE OF INOCULATED CALVES BY CONTACT WITH BPV INFECTED CALVES.

CALF	LESION TYPE *	
	at inoculation (see table 20) site.	at non-inoculation sites.
68882	A	C
68886	E	B
68888	B	E
68891	D	not applicable +
68893	C	B & E
TFBSC (i)	no lesions	not applicable +
TFBSC (ii)	no lesions	no lesions
TFBMC	no lesions	no lesions
FBSC	no lesions	E
FBMC	no lesions	no lesions

* Lesion type described in Materials & Methods as A -- E.

\+ These calves were not challenged by contact but were kept isolated.

even teat fibropapilloma without a papillomatous surface, still show papillomatous changes around the circumference of the lesion. There are, however, occasional lesions which cannot be distinguished grossly or histologically from anogenital fibropapilloma except for their anatomical site. Teat fibropapillomas are grossly and histologically indistinguishable from the normal cutaneous fibropapillomas reported in D/ below, again, except for anatomical site of occurrence. Microscopically, fibropapillomas are characterised by hyperkeratosis, acanthosis and a concurrent dermal fibroma, intimately involved with a disorganised and sometimes absent stratum basale in early lesions. On occasions, this leads to areas of ulceration - noticed as spontaneous bleeding on calf 68893 at week 12 post inoculation. The early lesions also show areas of acanthosis with a thickening of the stratum granulosum, though less pronounced than in A or B above. Intranuclear inclusions (basophilic) are rarely seen in early lesions with their first appearance 10 weeks post inoculation becoming more common with advancing time. As lesions mature, acanthotic epithelium is thrust upwards by connective tissue papillae composed entirely of fibroblastic cells with numerous vascular channels. The first evidence of attempted rejection was noticed (table 22) in calf 68893 14 weeks post inoculation, became more pronounced at 16 - 18 weeks with the accumulation of large numbers of lymphocytes but reduced at week 20 without successfully affecting the progressive development of large fibropapillomas. The presence of developing fibropapillomas derived from teat lesions and obvious, though unsuccessful, evidence of

rejection, did not prevent calf 68893 developing both papillomas and anogenital fibropapillomas as a result of the contact challenge part of the experiment.

D) Figure 15 shows the histological appearance of both the lesions from which BPV was extracted and those produced by transmission to calf 68891 (table 22). Macroscopically, cutaneous fibropapilloma are indistinguishable from those previously described in the literature (Bagdonas and Olson, 1953). Unlike teat fibropapilloma they are rarely subjected to sufficient regular trauma to cause partial or complete loss of their papillomatous surface. The lesions are generally indistinguishable from those described under C/ above but can be readily separated grossly and histologically from anogenital fibropapillomas by the presence of a pronounced papillomatous surface with frond length > 2mm. On transmission, calf 68891 (table 22) first showed fibropapilloma at week 8 post inoculation with the accompanying presence of small areas of lymphocyte accumulation. By week 14, the fibropapilloma had been rejected following the infiltration of large masses of lymphocytes with associated necrosis and cell death at week 10. Calf 68891 was held in isolation during the experiment, and it did not develop lesions on any skin site other than the inoculation sites.

E) Figure 16 shows the histological appearance of both the lesions from which BPV was extracted and those produced by transmission to calf 68886 (table 22). Macroscopically, the lesions present as large, sessile or more commonly pedunculated cauliflower -

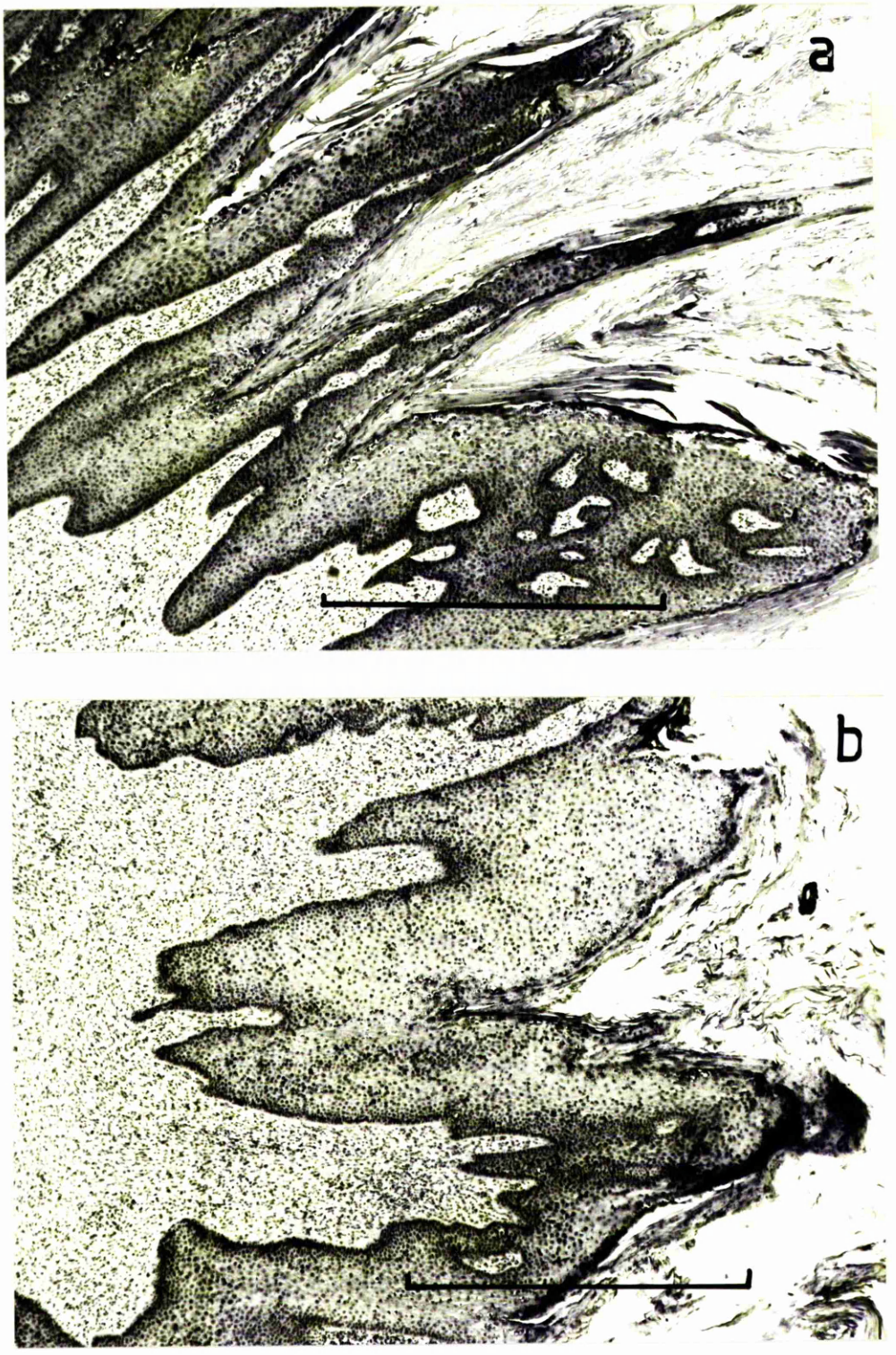


FIGURE 15 - (a) Histological appearance of cutaneous fibropapilloma from which BPV was extracted (b) Transmitted lesion showing fibropapilloma on skin of calf 68891. These lesions are not histologically separable from those illustrated in figure 14.
Stain H & E. Bar = 1mm.

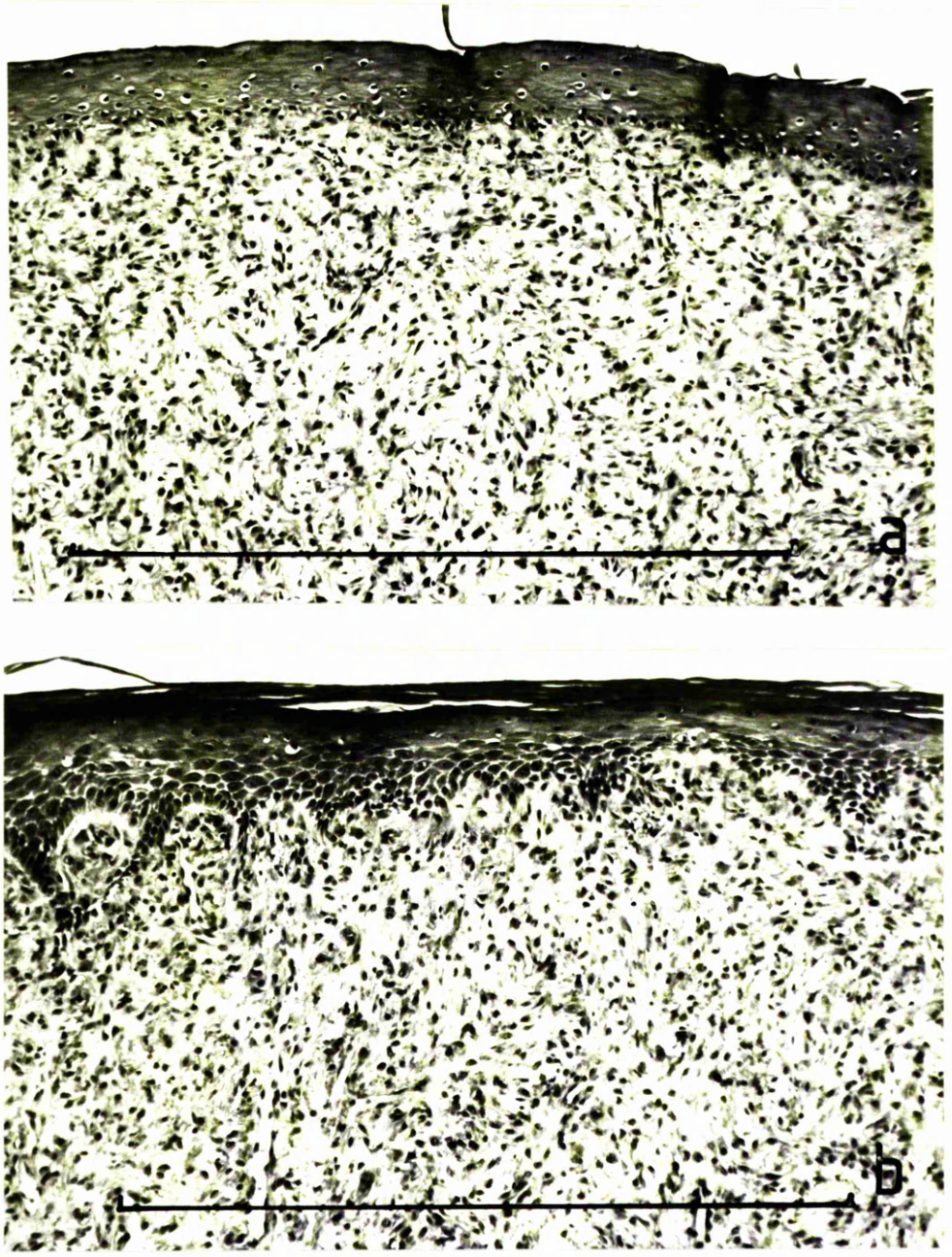


FIGURE 16 - (a) Histological appearance of ano-genital fibropapilloma from which BPV was extracted (b) transmitted lesion showing fibropapilloma on skin of calf 68886. These lesions are readily separable from other fibropapillomas by the absence of fronds > 2mm long and by their cauliflower-like appearance. Note the relative appearance of the epithelium when compared with other lesions illustrated here. The major appearance is that of a fibroma with secondary epithelial changes.
Stain H & E. Bar = 1mm.

like lesions. Papillomatosis is reduced or absent (fronds < 2mm) giving the appearance in early lesions of a smooth surface. On incision, the lesion comprises a whitish, fleshy fibroma covered by a thin epithelial layer which in later lesions may become slightly thickened. Histologically, the lesions are characterised by the development of a rapidly growing fibroma intimately involved with the indistinct stratum basale of an otherwise normal epithelium. Other dermal structures are pushed laterally and appear in concertina form adjacent to the main lesion. Epithelial rete bridges and hair sheaths become thin and give the appearance of arborisation due to the distortive effects of the fibroma. Cells in the fibroma are plump fibroblasts with open oval nuclei often containing several nucleoli. Pronounced capillary infiltration is a feature, accompanied by the occasional appearance of red blood cells between tissue spaces. In the mature lesion, slight hyperkeratosis with moderate acanthosis and intermittent parakeratosis are seen. Occasionally intranuclear inclusions are visible, but not as marked as in B/ above. On transmission (table 22) lesions were first noticed 6 weeks post inoculation accompanied by small accumulations of lymphocytes. By week 10, large areas of the fibroma contained massive accumulations of lymphocytes, with adjacent evidence of necrosis and cell death. By week 12, both the epithelial and fibromatous components were rejected, replaced by apparently normal epithelium and scar tissue. The development and regression of fibropapillomas on calf 68886 did not prevent the development and persistence of papillomas as described under B/above on the same animal.

F) Calves inoculated with control FBMC and FBSC and those inoculated with BPV transformed (T) FBSC and TFBMC developed no visible lesions at any inoculation site in the six months prior to the challenge section of the experiment. Biopsies at fortnightly intervals failed to show histological evidence of inoculated cell growth. The first biopsy at 2 weeks indicated the presence of fibroblastic cells at both FBSC and TFBSC carbon labelled inoculation sites.

3.3.2 Challenge

A. By Contact

Between two and eight weeks post inoculation, all calves (except 68891 and TFBSC (i) listed tables 23 and 24) were held in one pen. Weekly examinations revealed the transmission of lesions classed as B, C and E to non-inoculation sites (mainly the head). Table 23 lists the lesion types seen during this section of the experiment. The two transformed cell inoculated calves (TFBSC (ii) and TFBMC) did not develop lesions, while one (FBSC) of the two control cell inoculated calves developed lesions indistinguishable from E described above. Lesions B & E were each transmitted to two calves while lesions C was transmitted to one calf. Lesion A was not transmitted by contact to any calf and it should be noted that approximately half of the first inoculation scarification lines using BPV from lesion A on calf 68882 failed to develop lesions. Total lesion size has never been seen to exceed 1 cm in diameter either under natural conditions on the bovine teat or experimentally. Therefore a four plus rating as on tables 22 and 24 was not a reasonable

expectation for lesion A.

B. By Scarification

Table 24 lists the results of challenge of each calf by scarification using BPV from five lesion types (A - E). The results can best be summarised according to individual challenge sites. When challenged with BPV from lesion A, only one of the virus inoculated calves developed lesions - calf 68891 which had received virus from lesion D and had been kept in isolation. One of the cell inoculated calves failed to develop lesions, the isolated calf TFBSC (i). Challenge with BPV from lesion B showed a similar response with only virus inoculated calf 68891 and cell inoculated calf TFBSC (i) showing responses different to the others. Challenge with BPV from lesion C/ again showed the susceptibility of calf 68891 though with a reduced response with delayed development of small lesions <1 cm by week 12 post challenge. While lesion C/BPV challenged, cell inoculated calves showed variable responses. TFBSC (i) and TFBSC (ii) failed to develop lesions while TFBMC developed small early lesions which rapidly regressed. FBSC and FBMC control calves both developed lesions, though FBSC showed a reduced response in delayed development and small size lesions at week 12. Challenged with BPV from lesion D, three of the five virus inoculated calves responded with the early development and rejection of small (<1 cm) lesions while two (68886 and 68893) showed no response. Two of the three transformed cell inoculated calves developed a reduced response with early development of smaller lesions and early rejection. One transformed cell inoculated calf

TABLE 24 - CHALLENGE OF INOCULATED CALVES BY SCARIFICATION WITH BPV EXTRACTED FROM DIFFERENT LESION TYPES.

CALF	TIME (X)	PRIOR LESIONS	CHALLENGE SCARIFICATION SITE LESION TYPE *															AP
			A			B			C			D			E			
Time (Y)			5	10	12	5	10	12	5	10	12	5	10	12	5	10	12	AP
68882	13	AC	+			+			+			~~III~~	+		+			
68886	13	EB	~~II~~	+		~~II~~	+		+			+			+			
68888	13	BE	+			+			+			~~III~~	+		+			✓
68891	23	D	+	~~III~~	~~III~~	+	~~III~~	~~IIII~~	+	~~II~~	~~III~~	~~III~~	~~III~~	+	~~III~~	~~III~~	~~III~~	
68893	13	CBE	+			+			+			+			+			
TFBSC (i)	23	n.l	+			+			+			II	+		+			
TFBSC (ii)	36	n.l	~~II~~	~~III~~	~~III~~	~~II~~	~~III~~	~~IIII~~	~~II~~			~~III~~	~~III~~	+	~~II~~			✓
TFBMC	36	n.l	~~II~~	~~III~~		+	~~II~~	~~IIII~~	~~III~~	~~II~~		~~III~~	~~II~~		+			
FBSC	36	E	+	+	~~III~~	~~II~~	~~III~~	~~IIII~~	+	~~II~~	~~III~~	~~II~~	+		~~II~~	+		
FBMC	36	n.l	~~III~~	~~III~~	~~III~~	~~III~~	~~IIII~~	~~IIII~~	~~IIII~~	~~IIII~~	~~IIII~~	~~III~~	~~IIII~~	~~IIII~~	~~IIII~~	~~IIII~~	~~IIII~~	✓

* Lesion types A - E described in Materials and Methods
X Time in weeks between first inoculation and challenge
y Time in weeks following challenge by scarification
n.l = no lesions
T - Transformed cells as in TFBSC or TFBMC
+, ~~II~~, ~~III~~, ~~IIII~~ explained at bottom of table 22.
AP - alimentary papillomas found at post-mortem examination (✓)

(TFBSC (ii)) failed to respond. One control calf (FBMC) showed complete susceptibility while the other, FBSC, which had responded to E in the contact challenge period, failed to respond to challenge by scarification. When challenged with BPV from lesion E, two calves responded. Calf 68891 showed early response with lesions of reduced size while the control cell inoculated calf FBMC was completely susceptible. Calf FBSC had developed lesion E fibropapillomas during the contact challenge period.

3.3.3 Electron Microscopy

Thin section examination failed to detect the presence of BPV in BPV infected FBMC and FBSC at any time beyond the first passage. E.M. examination of negatively stained preparations of spent culture medium also failed to detect BPV. All virus preparations used for animal inoculation, and scrape samples of transmitted lesions on calves showed the presence of BPV in negatively stained preparations when examined under the E.M.

3.3.4 Post Mortem Examination

Detailed post mortem examination detected the presence of upper alimentary papillomas in three of the eight calves that were not kept under isolation.

Calf 68888 showed papillomas on the soft palate, tongue and oesophagus (figure 17 (b))

Calf FBMC showed papillomas on the buccal mucosa and the mucosa lining the internal nares (figure 17 (a)) and the mucocutaneous junction.

Calf TFBSC (ii) showed papillomas on the oesophagus.

Calf 68888 additionally showed an erosive, granulomatous lesion on the ruminal wall and cystic hyperplasia of the gall bladder wall.

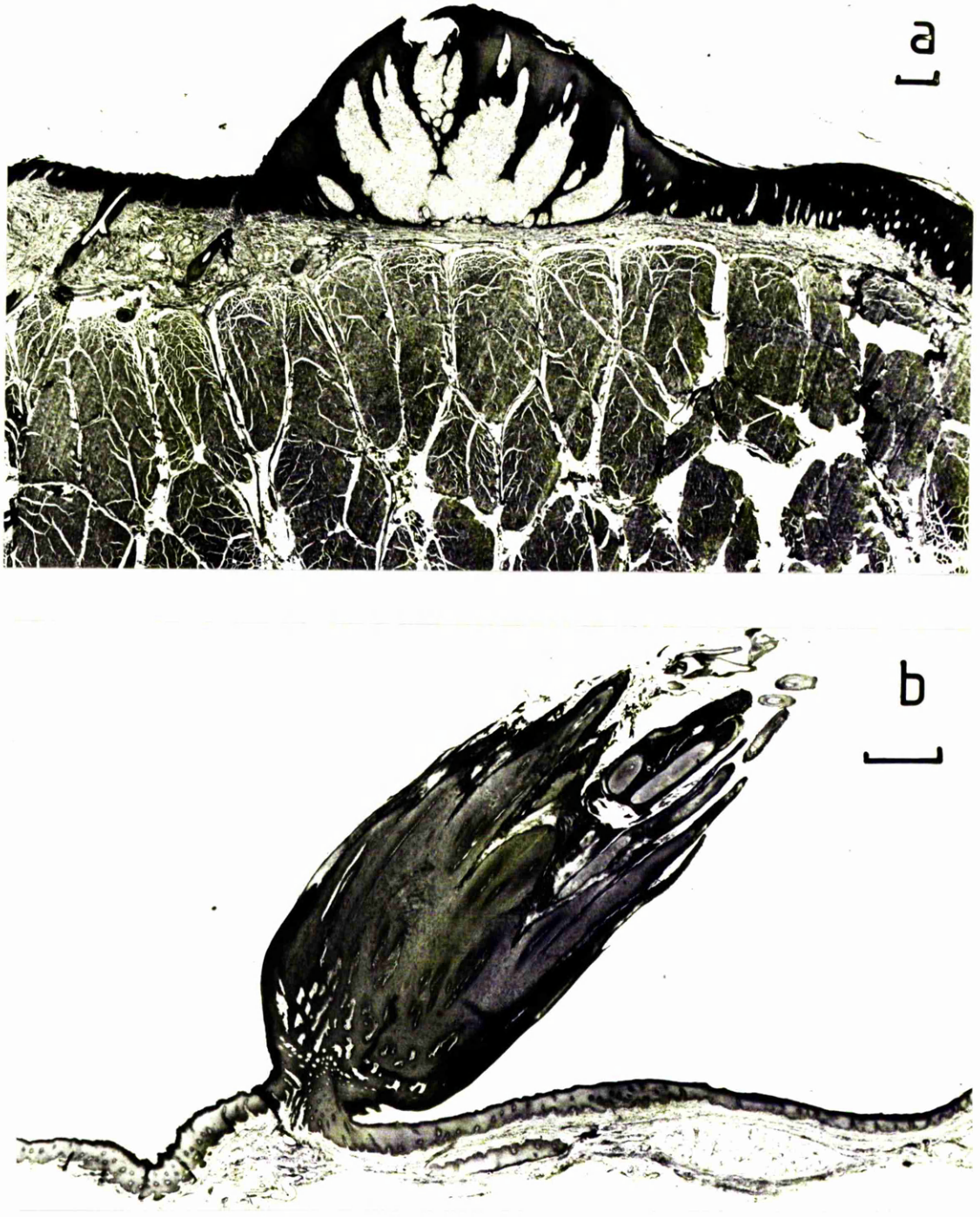


FIGURE 17 - (a) Fibropapilloma present at mucocutaneous junction of oral cavity of calf FBMC, this calf had fibropapillomas and papillomas present both within the oral cavity and on the external cutaneous surface of the face. (b) Papilloma on wall of oesophagus of calf 68888, this calf also had papillomas on the palate, tongue and oropharynx.
Stain H & E. Bar = 1mm.

Haematological examination of sequential blood samples was unremarkable. Appendix C records the haematological results in detail.

3.4 Discussion

The isolation of BPV from and the detection of three morphologically separable teat lesions have been reported previously (Chapter 2). However the transmission of these lesions to other parts of the integument experimentally represents a new finding. On transmission, BPV extracted from one lesion type produced lesions of the same type on another anatomical site. The existence of papillomas and the extraction of BPV from them has been reported before (Barthold et al., 1974), however attempts at experimental transmission and immunological characterisation of the virus using methods standard for BPV were unsuccessful. Barthold et al., (1974) noted the tendency of tumours to persist, rather than regress which occurred with calf 68888 (table 22) in the experiments reported here. Similarly, papillomas were reported to occur concurrently or subsequently on animals with typical fibropapillomas. This chapter also records the development and regression of fibropapillomas on calves 68888 and 68882 showing concurrent papillomas and rice grain lesions respectively which did not regress.

Rice grain lesions are macroscopically and histologically similar to focal epithelial hyperplasia (Praetorius - Clausen, 1972) which occurs in the human oral cavity, and in which a papova virus has been detected (Praetorius-Clausen and Willis, 1971). The lesions transmitted to calf 68882 persisted from week 6 to week 20 post inoculation and rejection was not accompanied by the massive lymphocyte infiltration associated with regression of fibropapillomas. Of the three fibropapilloma transmitted calves, only one inoculated with BPV from anogenital fibropapillomas produced lesions macroscopically and histologically separable from the other two, confirming earlier observations on field case lesions. However,

both the anogenital and the cutaneous fibropapillomas showed an early and successful rejection response at weeks 8 - 10 post inoculation while calf 68893 showed a delayed rejection response 16 - 18 weeks post inoculation which was unsuccessful resulting in the persistance of lesions till post mortem at 24 weeks. The absence of E M detectable BPV in transformed or control FBSC and FBMC was confirmed in vivo by the absence of lesion development during the six months post inoculation.

Combining the results of challenge by contact and scarification, the following points may be made.

1) Animals showing papillomas or rice grain lesions remain susceptible to transmission of fibropapillomas. Rejection of fibropapillomas is not accompanied by rejection of papillomas or rice grain lesions.

2) Animals showing either papillomas or rice grain lesions are immune to challenge by scarification of BPV from both lesion types (calves 68882, 68886, 68888 and 68893). However animals not showing either lesion type remain susceptible to challenge by BPV from both lesion types (calves 68891, TFBSC (ii), TFBMC, FBSC, FBMC). Only one calf, inoculated with TFBSC (i) showed resistance to challenge by both BPV isolates without showing prior lesions.

3) Within the fibropapilloma group (lesions C, D, E), prior infection by BPV from anogenital fibropapillomas (E) was followed by resistance to challenge by scarification of BPV from lesion C in calves 68882, 68886, 68888 and 68893 (which had earlier developed lesion C and E following inoculation and contact challenge) Calf FBSC which developed lesion E in the contact challenge stage showed a reduced response to lesion C scarification challenge. Calf FBMC was completely susceptible to challenge by scarification of all fibropapilloma derived BPV (C, D, E). Of the three calves which received lesion D transformed cells, TFBSC (i) and TFBSC (ii) were

resistant to challenge by both lesion C and E while calf TFBMC showed resistance to lesion E and a reduced response to lesion C. Finally isolated calf 68891 which developed lesion D was susceptible but with a reduced response to BPV from all three fibropapilloma lesions. However response to lesion D was more rapidly rejected. Challenge by lesion D BPV scarification resulted in no response in calves 68886, 68893, TFBSC (i) and FBSC and a reduced response in all other calves except FBMC which remained fully susceptible.

4) Control cell inoculated calves, FBSC and FBMC, were susceptible to challenge stages of the experiment. Transformed cell inoculated calves were either resistant or developed a reduced response to challenge by BPV from the three fibropapilloma lesion types.

These experiments suggest that there are two broad BPV groups, BPV from papilloma and rice grain lesions and BPV from fibropapillomas. Within these two groups there are gross, microscopic, developmental differences which are reproducible by experimental transmission. Animal resistance to one broad group does not influence resistance to the other group. The use of cells transformed in vitro by fibropapilloma derived BPV increases resistance to challenge by fibropapilloma derived viruses but not BPV extracted from papilloma or rice grain lesions.

These findings may partly explain the widely varying results of BPV vaccination reported in the literature. (review by Olson et al.1969). The finding of alimentary papillomas in three of eight in contact calves at post mortem was unexpected although the suggestion has been made earlier (Chapter 2; Jarrett et al., 1978) that teat papillomatosis may lead to alimentary papillomas. A survey of over 7,000 cattle showed an overall prevalence of alimentary papillomas of 19% (Jarrett et al., 1978). The number of animals used in the experiments reported in this paper are small and the results must necessarily be treated as preliminary.

FIGURE 18 - Photograph showing macroscopic difference between transmitted anogenital fibropapilloma (a) and teat fibropapilloma (b) calf FBMC. Note smooth surface of (a) as contrasted with papillomatous surface of b) Bar = 2cm.

Further in vivo and in vitro work using BPV from single case, single type lesions must be done to fully elucidate the differences between various BPV extracts.

SUMMARY

Bovine papilloma virus (BPV) was extracted from five cattle each affected with only one of five morphologically separable lesion types. When inoculated into experimental calves either by scarification or intradermal injection, the BPV extracts produced lesions macroscopically and microscopically similar to those from which individual extracts were made. Foetal bovine cells, transformed in vitro with BPV, failed to produce fibromas, fibropapillomas or papillomas when inoculated into experimental calves. When virus and BPV transformed cell inoculated calves, were challenged with the five original BPV extracts, a differential immunity was demonstrated, while control calves were susceptible to all extracts. Post mortem examination revealed the presence of upper alimentary tract papillomas in three of eight calves forming one group. These results suggests that different BPV's exist, causing morphologically separable lesion types. There may be additional BPV variants causing fibropapillomas of the teat and anogenital regions of cattle. The inoculation of BPV transformed foetal bovine cells conferred a relative immunity to later challenge with some but not all BPV extracts.

CHAPTER 4

IN VIVO EFFECTS OF BOVINE PAPILLOMA VIRUS: THE TUMORIGENICITY IN ATHYMIC NUDE MICE OF BOVINE PAPILLOMA VIRUS AND BPV TRANSFORMED FOETAL BOVINE SKIN CELLS.

4.1 Introduction

Bovine Papilloma Virus (BPV) has been shown in vitro to transform bovine cells (Black et al., 1963; Thomas et al., 1963) and primary embryonic cell cultures from C3H/eB, C57/BL and BALB/c mice (Thomas et al., 1964). In vitro transformation by BPV has been reported using foetal bovine cultures derived from skin, palate, meninges and conjunctiva (Chapter 7). In vivo, BPV is tumorigenic in a wider range of species than other papilloma viruses. Intracranial injection of BPV produces meningiomas in calves and fibroblastic intracranial neoplasms in the hamster (Gordon and Olson, 1968; Robl et al., 1972). Subcutaneous injection of BPV produces fibromas in C3H/eB mice and hamsters (Friedman et al., 1963; Cheville, 1966; Robl and Olson, 1968), and intradermal injection of BPV in the horse induces a connective tissue tumour similar to equine sarcoid, naturally occurring cases of which have been shown to contain BPV virus-specific DNA sequences (Olson and Cook, 1951; Lancaster et al., 1977). Several reports suggest that BPV is transmissible to man, (Schultz, 1908; Frenz, 1941; Grenier et al., 1976) and this is supported by the observation that persons in certain occupations such as meat handlers are at high risk of developing warts (Perel and Lumpkin, 1976). This chapter describes the results of inoculation of BPV, BPV transformed cells and untransformed cells into nude mice.

4.2 Materials and Methods

4.2.1 Cells

The skin of a 60 cm. near term bovine foetus was lightly scraped with a sterile scalpel blade. Collected

scrapings were placed in an 8oz. medical flask in 20 ml basal Eagle's medium (BME) with 20% added foetal bovine serum (FBS). The first subdivision of cells occurred 10 days later, and subsequently at weekly intervals using BME with 15% FBS. Cultures in the fourth passage were infected in suspension with $10^{-3.5}$ dilution of BPV (highest dilution showing 100% transformation - see later) as described elsewhere (Chapter 7) and the resultant transformed cultures from passage 12 were used to inoculate nude mice. Parallel untransformed cultures at passage 12, derived from the same foetus served as controls for nude mouse inoculation. Paired BPV transformed and control foetal bovine skin cell (FBSC) cultures were maintained in vitro throughout the experiment. At regular intervals, a sample of both cell cultures was grown on a glass slide placed in a 10 cm. diameter plastic petri dish for examination to check maintenance of transformed phenotype.

4.2.2 Examination of Cells

Cells grown on glass slides were stained with Giemsa after methanol fixation as described elsewhere (Chapter 7). Cells were examined macroscopically, and under a Leitz light microscope.

4.2.3 Virus

BPV was extracted using fibropapillomas collected from a single field case infected with cutaneous fibropapillomas (Chapter 3). Techniques used for extraction have been described elsewhere (Chapter 7).

4.2.4 Transformation Assay

Paired samples of transformed and control FBSC cultures were assayed using BPV described above to determine the Log_{10} Transforming Dose 50% (Log TD_{50}) (Chapter 7).

4.2.5 Nude Mice

Sixty five-BALB/c random outbred cross 3-4 week old nude mice were purchased from Olac Pty Ltd. The mice were randomly divided into three groups of 20 and one group of five animals. Within 24 hrs. after arrival animals were inoculated with FBSC, BPV transformed FBSC and BPV. One 0.1 ml inoculation was made subcutaneously over the rib cage on each side of each animal. All inoculations contained 10mg/ml of filtered, sterilised activated charcoal which was added, to mark the site of injection. Cell inoculations were carried out using thrice PBS rinsed suspensions in PBS at a concentration of 10^7 cells/ml. Thus all animals received 10^6 cells per inoculation. The virus suspension was a 10% w/v homogenate from single case papillomas clarified by centrifugation for 30 min at 10,000 r.p.m. in an SW27 rotor, with added 10 mg/ml activated charcoal. Five nude mice were not inoculated and served as untreated controls.

4.2.6 Histopathology

Because of the relatively short life span of nude mice held under normal laboratory conditions, animals were either sacrificed in extremis or died naturally. Each nude mouse was subjected to a detailed post-mortem examination. Internal organs were removed through a ventral midline incision. The whole carcase and organs were fixed for one week in 10% formol saline, before fixed samples were removed for further processing. Irrespective of treatment, each animal provided a section of inoculation site, lung, liver, spleen, kidney and any other tissue showing gross pathological changes. Sections were routinely stained with H & E and with appropriate special stains when the need arose.

4.2.7 Electron Microscopy

Tumours from ten nude mice were excised and immediately placed in 10% w/v PBS. Tumour tissue was homogenised using a Silverson blender and subjected to differential centrifugation outlined for virus preparation elsewhere (Chapter 7). Samples of crude homogenate were negatively stained with phosphotungstic acid and examined for the presence of BPV under the electron microscope. Samples from $CsCl_2$ gradient purified suspensions around specific gravity of 1.36 were similarly examined.

All used medium from transformed and control FBSC cultures was separately collected, precipitated at $4^{o}C$ with 50% added saturated $(NH_4)_2$-SO_4, re-suspended in PBS and again subjected to differential centrifugation before $CsCl_2$ gradient purification and EM examination for the presence of BPV.

Transformed and control FBSC cultures were grown on 5 cm plastic petri dishes for three days, the medium was removed and cells were fixed in glutaraldehyde in situ. After 24 hrs. cells were removed using a rubber policeman and further processed for thin section electron microscopy. Samples of one tumour produced by transformed FBSC inoculation, was fixed in glutaraldehyde and further processed for thin section electron microscopy.

4.2.8 Attempts at In Vivo and In Vitro Propogation of Tumours.

Six tumours were excised under sterile conditions and rinsed three times in BME. Three of the tumours were cut up into small pieces and placed in an 8oz. medical flask with 20 ml of BME with 20% added FBS. The other three tumours were individually treated for three minutes in a Stomacher before similarly being placed into 8oz. medical

flasks with 20 ml BME with 20% added FBS. Bottles were examined at weekly intervals for evidence of cell growth.

Two tumours were excised under sterile conditions, treated in a Stomacher for three minutes in 2 ml BME. The resultant cell suspension was inoculated subcutaneously into five nude mice after addition of 10 mg/ml of activated charcoal. Animals were observed for evidence of tumour development.

4.3 Results

Table 25 summarises the effects of various treatments on nude mice. No significant differences occurred between the mean survival times of transformed FBSC, FBSC injected and control animals. However the mean survival time of the BPV infected nude mice was significantly lower ($P < 0.01$). The distribution of major lesions on an organ by organ basis was similar in all three groups.

All nude mice inoculated with BPV transformed FBSC showed cell proliferation out of the carbon labelled injection sites. 45% of these tumours resulted in invasion of adjacent tissues (Figure 20). Two animals in the untransformed FBSC inoculated group showed some cell outgrowth, from injection sites, but this was limited and non-invasive. One BPV inoculated animal showed an inflammatory response at the injection site, however no BPV inoculated animal showed any evidence of tumour production.

Figure 19 illustrates the differences between the three treatment groups graphically. There was a rapid growth of tumours for the first 30 days post inoculation. Thereafter, the tumours progressively changed to produce well differentiated fibromas (see Figure 20).

TABLE 25: RESULTS OF S/C INOCULATION OF BPV, BPV TRANSFORMED FBSC AND UNTREATED FBSC INTO NUDE-MICE.

GROUP	A	B	C	Control
Number of Nude Mice inoculated with	20 10^6 BPV transformed FBSC.	20 10^6 FBSC	20 10^{-1} BPV	5 nothing
Mean survival time $\pm$ S.E. (days)	37.8 ± 4.4	33.6 ± 3.0	21.9 ± 2.5	40.2 ± 8.7
Number animals showing growth at injection site	20	2	1	0
Number of animals showing tumour invading surrounding tissues	9	0	0	0
Organ involved as major cause of death.				
Liver	45%	35%	40%	
Lung	40%	45%	40%	
Mixed	5%	20%	20%	

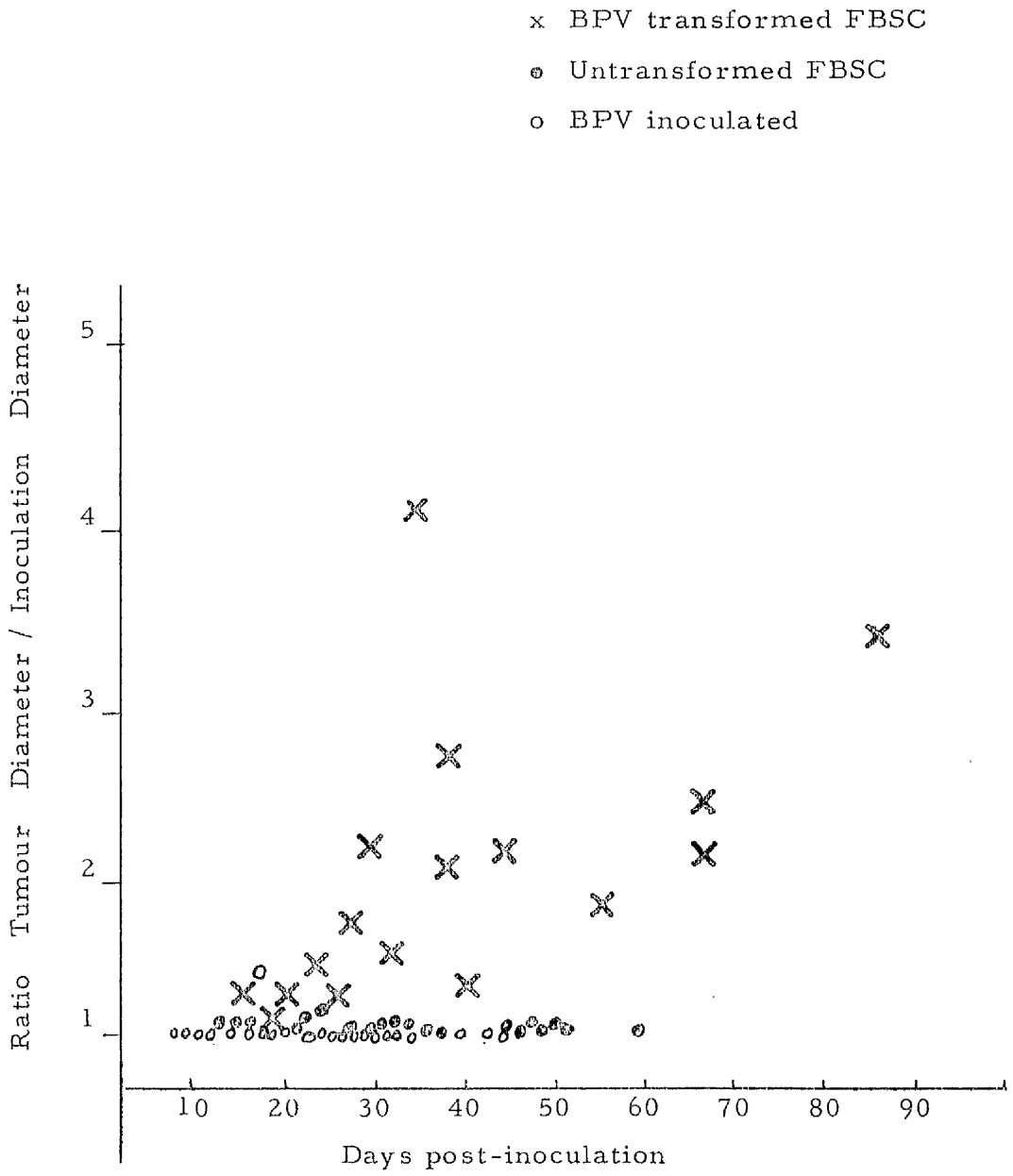


FIGURE 19 : Nude mouse tumour growth related to time post-inoculation.

Figure 20 illustrates these features with low power photomicrographs of tumours from two animals which died or were sacrificed at different times post-inoculation.

Cell types in the original inoculum and in the earlier, rapidly growing tumours were similar and largely comprised fibroblasts as well as a smaller proportion of epitheliod cells which, when using special stains, resembled keratino cytes. High power photomicrographs of these cell types appear in Figure 21.

Detailed histopathological examination of all major internal organs failed to detect any sign of tumour metastases or primary tumour development. Electron microscopic examination of homogenised transformed FBSC, FBSC and ten nude mouse tumours failed to detect the presence of BPV particles in negatively stained preparations. EM examination of $CsCl_2$ purified spent medium collected from parallel cell cultures, negatively stained, also failed to detect BPV. Thin section electron microscopy failed to detect BPV particles in transformed FBSC, FBSC and nude mouse tumours.

Attempts to propagate nude mouse tumours in vitro and in vivo were not successful irrespective of the treatments given.

Repeated assay of the same virus preparation on untransformed FBSC resulted in no significant differences in Log_{10} TD 50 over thirty passages. Paired transformed FBSC cultures retained their transformed characteristics for twenty-six passages and then progressively lost their transformed appearance. Attempts to retransform these revertant cultures by BPV infection were unsuccessful.

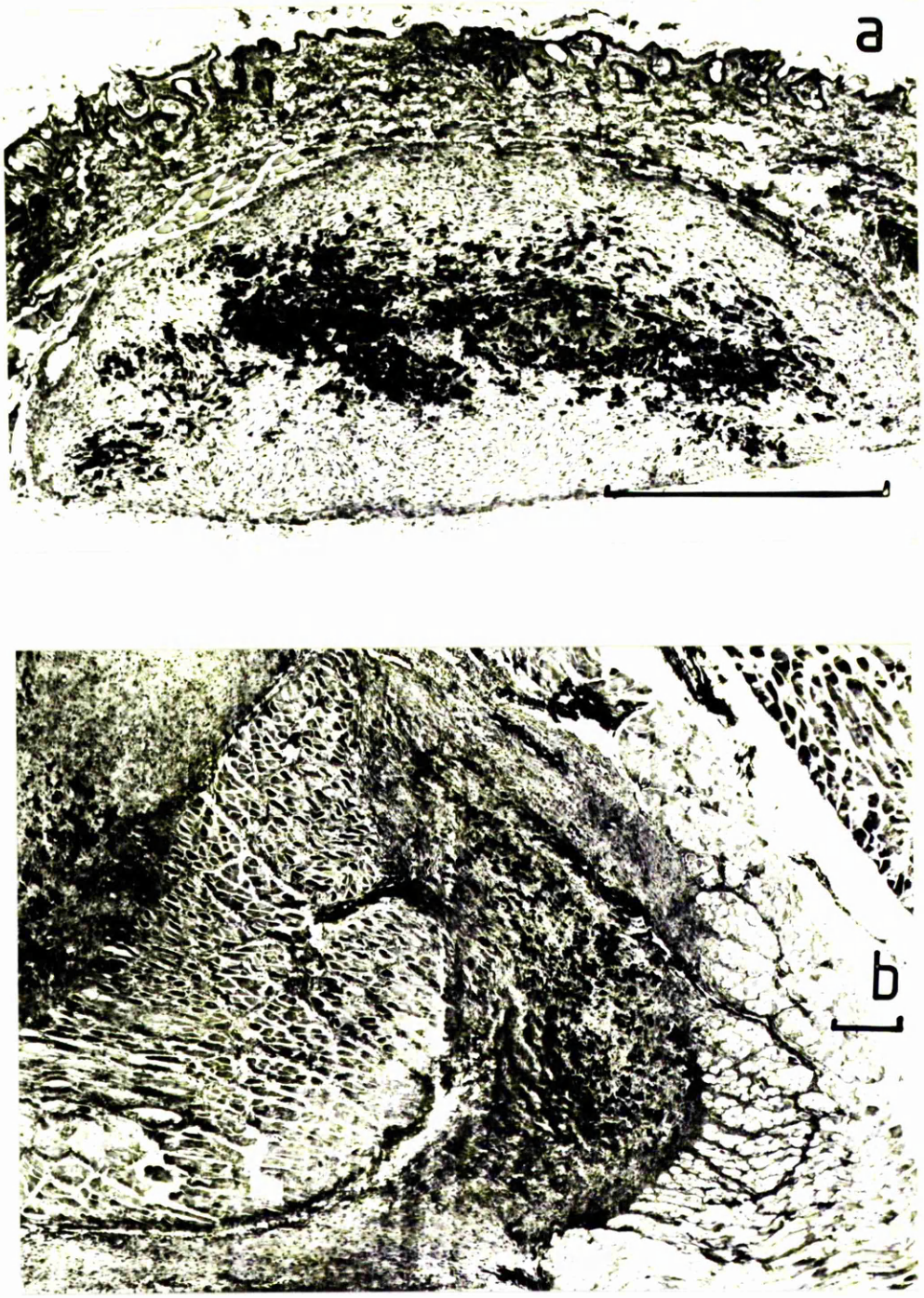


FIGURE 20 Low power photomicrographs of BPV-transformed induced nude mouse tumours a) 16 days post inoculation b) 30 days post inoculation. Stained with MSB. Bar = 1 mm.

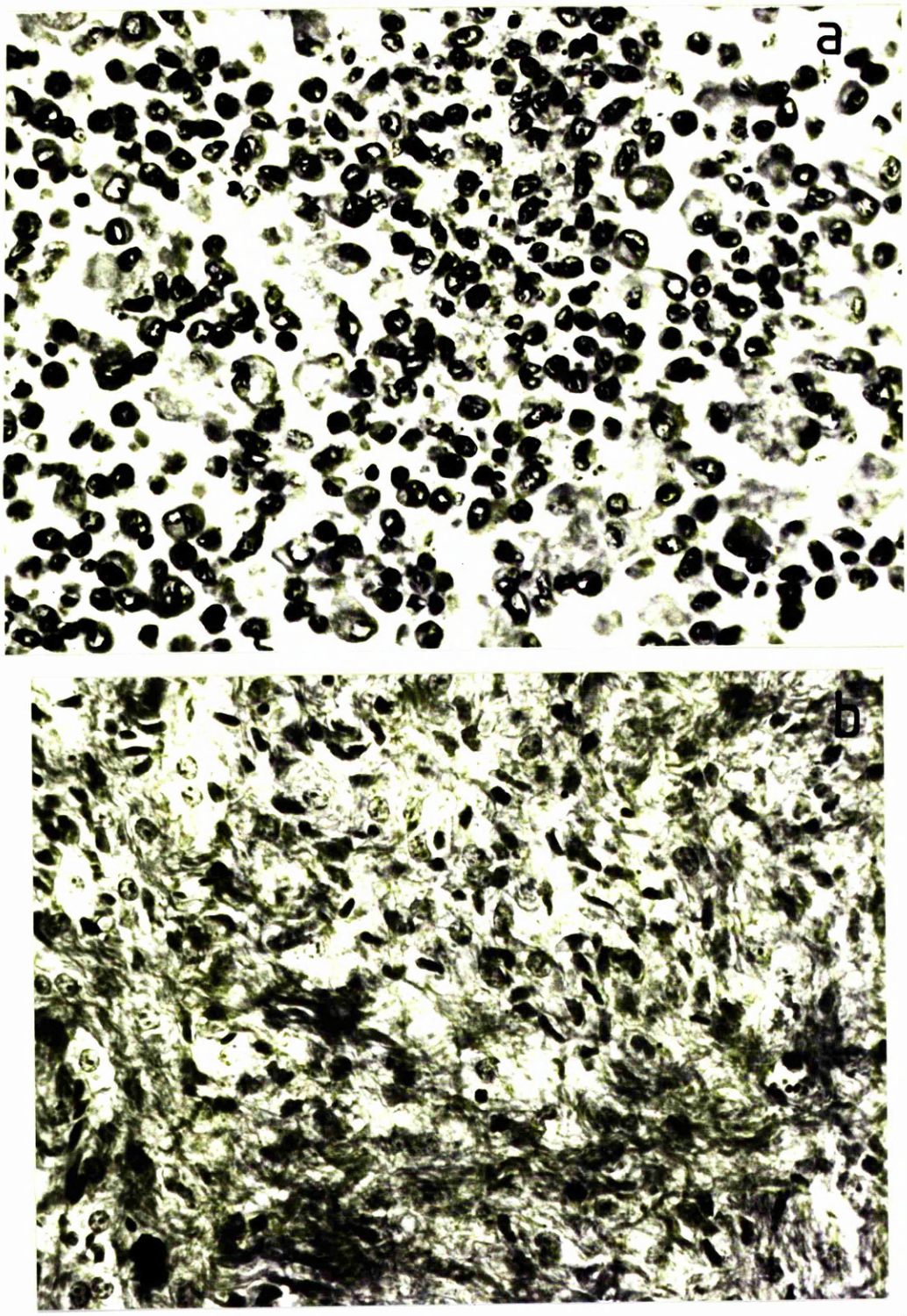


FIGURE 21

a) High power photomicrograph of in vitro transformed cells, fixed in situ. Stained with MSB. Magnification x 500.

b) High power photomicrograph of nude mouse tumour induced by transformed cells. Stained with MSB. Magnification x 500.

4.4 Discussion

Cell cultures transformed by other DNA viruses have been shown to be tumourigenic in nude mice. Human cells are transformed by cytomegalovirus (Culp et al., 1971); Epstein-Barr virus - specific nuclear membrane antigen positive tumour cells from nasopharyngeal carcinoma in man have been passaged in nude mice (Vuillaume et al., 1977). Adenovirus - transformed cell lines produce non-metastasising tumours which may show invasion of local tissues (Gallimore et al., 1977). Cells transformed by other members of the papova virus group have also been reported to produce tumours in nude mice. SV 40 virus transformed mouse foetal brain cells (Benda et al., 1977); human-mouse somatic cell hybrids from mouse peritoneal macrophages and SV40 transformed human cells (Aden et al., 1977); SV40 transformed human cells (Koprowski and Croce, 1977); polyoma transformed mouse cells (Grady et al., 1977); and MMV transformed nude mouse kidney cells (Costa et al., 1977) have produced tumours when inoculated into nude mice. However, there are no reports in the literature of papilloma virus transformed cells producing tumours in nude mice. The only other report of bovine tumour cell induction of tumours in non-irradiated nude mice involved the inoculation of cells from bovine ocular squamous carcinoma (Hoffman et al., 1977). The results reported in this paper therefore represent the first time a papilloma virus transformed cell culture has induced tumours in nude mice.

The nude mouse is surprisingly resistant to tumour growth. Allogenic and xenogenic tumours grow better and metastasise more readily in the strain or species of origin than in the nude mouse. In fact, tumours in nudes may actually regress after temporary growth (Prehn, 1976). All of the afore-mentioned nude mouse tumours induced by other papova virus transformed cells failed to metastasise. The findings in this chapter are similar.

BPV has been reported to cause tumours in C3H/eB mice in vivo, and to transform cells from C3H/eB, C57/BL and BALB/c mice in vitro (Thomas et al., 1964). No information is available in the literature as to the time delay between BPV injection and tumour production in C3H/eB mice. However, in the relatively short time between BPV injection and death in the work reported here, no tumour development was detected either at the site of inoculation or elsewhere in the nude mouse.

This chapter reports for the first time a BPV transformed cell culture which has reverted to a normal growth pattern indistinguishable from paired transformation sensitive control cultures. The revertant culture was not sensitive to transformation by BPV even though paired control cultures at the same passage number were. Revertant cultures have been reported for SV40 transformed cells of hamster, mouse and monkey origin and a number are resistant to SV40 re-infection (Culp et al., 1971; Wilson et al., 1976; Diamandopoulus et al., 1976). A viral-inhibiting factor has been demonstrated in polyoma transformed cells (Berebbi et al., 1977) which depended on cell contact.

The absence of BPV in used culture medium, transformed cells and mouse tumours confirms earlier findings on the behaviour of BPV as reviewed by Olson et al., (1969).

Previous evidence that tumour induction in nude mice could be enhanced by pre-treatment with irradiation (Prehn, 1976) suggests that further work on BPV transformed

cell tumour production in irradiated nude mice may provide useful additional information

4.5 Summary

Three groups of twenty athymic nude mice were inoculated subcutaneously - one with foetal bovine skin cells (FBSC) transformed *in vitro* by bovine papilloma virus (BPV); one with a suspension of BPV; and the third with untransformed FBSC. All transformed FBSC inoculated mice showed cell outgrowth from the inoculation site. Tumours grew rapidly at first, progressively invaded adjacent tissues and matured into well differentiated fibromas. No evidence of tumour regression was seen. Attempts to visualise BPV in tumours by negative staining and thin section examination under the electron microscope were not successful. Attempts to grow tumour cells *in vitro* and to serially transmit tumours failed. Parallel *in vitro* culturing of transformed FBSC showed a progressive loss of transformation characteristics and revertant cultures did not transform after reinfection with BPV. Paired untransformed FBSC cultures, however, remained sensitive to BPV induced transformation.

No tumours developed in untransformed FBSC and BPV inoculated groups, however the mean survival time of the BPV inoculated group was significantly less than the other groups.

CHAPTER 5 IN VIVO EFFECTS OF BOVINE PAPILLOMA VIRUS - THE EFFECTS OF INTRACEREBRAL INOCULATION OF BOVINE PAPILLOMA VIRUS AND BPV - TRANSFORMED CELLS IN EXPERIMENTAL CALVES.

5.1 Introduction

Bovine papilloma virus extracted from cutaneous fibropapillomas has been shown to cause fibroblastic neoplasms of the meninges when inoculated intracerebrally (Gordon and Olson, 1968). The isolation of BPV from alimentary papillomas was reported by Jarrett et al., 1978; and a detailed account of the isolation and transmission of BPV extracted from teat lesions appears in chapters 2 and 3. Chapter 7 reports that BPV extracted from teat rice grain lesions and teat papillomas does not transform foetal bovine cells in vitro, even though the cells used were transformation sensitive to BPV extracted from a pooled sample of alimentary tract lesions and single case sampled teat fibropapillomas. Sera from cattle infected with BPV from rice grain lesions and teat papillomas do not inhibit in vitro transformation by cutaneous fibropapilloma derived BPV while sera from cattle with fibropapillomas do (chapter 8). Some evidence presented in chapter 2 indicates physical and biochemical differences between BPV extracted from various skin and teat lesions.

This chapter reports experiments designed to detect differences between samples of teat, cutaneous and alimentary derived BPV in their ability to produce tumours following intracerebral inoculation. The finding that BPV transformed foetal bovine skin cells were tumourigenic in nude mice (chapter 4), but failed to produce tumours following intradermal, subcutaneous or submucosal inoculation in calves (chapter 3) made it important that the tumourigenicity of BPV transformed cells be tested intracerebrally in the bovine.

5.2 Materials and Methods

5.2.1 Experimental calves.

Six, one to two month old dairy cross calves were

randomly separated into two groups of three animals. One group was inoculated with cells while the other with virus. Each calf was anaesthetised using pentothal and maintained under halothane/oxygen supplied using an endotracheal tube.

Two sites on the frontal bone just above the junction of the right and left olfactory lobes with the cerebrum were prepared for surgery by shaving the skin and swabbing with Iodine mitis solution. Following a small 0.5cm skin incision, a 2mm medullary bone pin was used to drill a small hole to allow intracerebral inoculation. Two sites, one on the left shoulder and the other on the left hind leg were similarly prepared. These skin sites each received ten 0.1ml intradermal injections. The left cerebrum and shoulder sites were inoculated with either virus or transformed cells (see later) while the right cerebrum and hind leg sites received extracts of normal skin or control cell inoculations. All inoculations contained sterilised, filtered activated charcoal as previously described (chapter 3 and 4) to act as a marker for subsequent sampling.

At fortnightly intervals for the first 20 weeks post-inoculation all six calves were bled to provide serum and blood for haematological examination. At the same time, one intradermal inoculation site from the shoulder and hind leg was biopsy sampled under local anaesthesia using Xylocaine. Samples were fixed in 10% formol saline before being processed for further histopathological examination. Sections were routinely stained with Haematoxylin and Eosin and other special stains when appropriate.

Thirty-five weeks following inoculation, all six calves were sacrificed prior to detailed post mortem examination. Samples for further histopathological examination were processed as described above.

5.2.2 Virus

Three virus samples were used, one for each calf. (1) Calf 91 received a 10% w/v suspension of cutaneous fibropapillomas derived from a single field case. Control sites were inoculated with a 10% w/v suspension of normal skin from the same animal. (2) Calf 82 received a 10% w/v

suspension of cutaneous fibropapillomas which developed along scarification lines inoculated with BPV derived from a pooled sample of alimentary tract lesions. This sample was provided by Professor Jarrett. Control sites were inoculated with a 10% w/v suspension of normal bovine oesophagus. (3) Calf 84 received a 10% w/v suspension of single case derived rice grain lesions (sample RGB in chapter 2, table 21). Control sites were inoculated with a 10% w/v suspension of normal bovine teat.

5.2.3. Cells

Foetal bovine skin cells were established as described in chapter 7. Cells were infected in suspension with each virus sample (10^{-2} on-plate dilution). The alimentary and cutaneous fibropapilloma-derived BPV-inoculated cultures showed the typical transformation-associated changes described in chapter 7, but the teat rice grain lesion derived BPV inoculated cultures did not. Cells were BPV infected during their 4th passage and were harvested between passages 12-14 for calf inoculation. Parallel uninfected cultures from the same source were also harvested between passages 12-14 for control site calf inoculation. Cells were suspended using trypsin/versene, washed twice with PBS warmed to 37°C, and following centrifugation, resuspended in sufficient PBS to contain 10^7 live cells per ml. Calves 45, 81 and 83 received cells infected with BPV from cutaneous fibropapilloma, teat rice grain lesions and alimentary fibropapilloma respectively.

5.3 Results

Table 26 summarises the effect of intracerebral and intradermal inoculation of BPV and BPV transformed skin cells in experimental calves.

Table 26A The effect of intracerebral and intradermal inoculation of BPV and BPV-transformed skin cells in experimental calves.

Calf Number	91	82	74	45	81	83
Inoculated with *	CBPV	ABPV	TBPV	CTFBS	ATFBS	TIFBS
Lesions at intradermal inoculation sites	fibropapillomas	fibropapillomas	Rice Grain lesions	no lesions	no lesions	no lesions
Lesions at intracerebral inoculation sites	meningoma	meningoma	no lesions	no lesions	no lesions	no lesions

* CBPV - cutaneous fibropapilloma-derived BPV

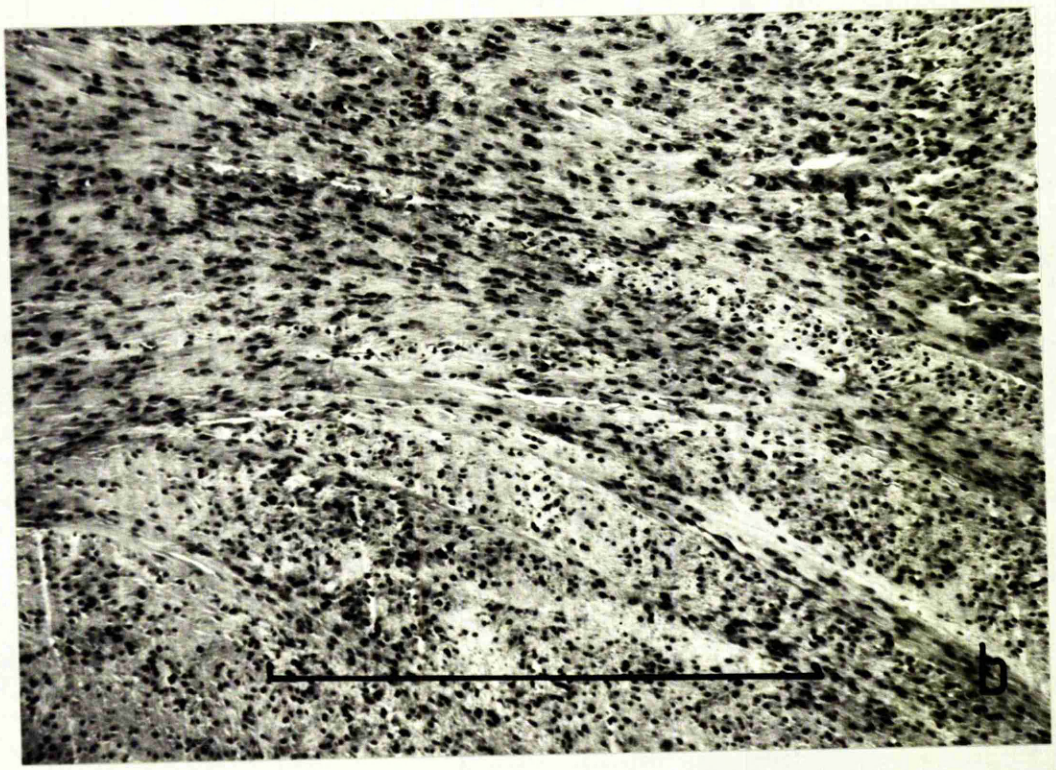
ABPV - alimentary lesion-derived BPV (see text for details).

TBPV - teat rice grain lesion-derived BPV

CTFBS - CBPV-transformed foetal bovine skin cells

ATFBS - ABPV-transformed foetal bovine skin cells

TIFBS - TBPV infected, untransformed foetal bovine skin cells


b

Calves showed lesions at the ten intradermal virus inoculated sites. Fibropapillomas developed on the cutaneous fibropapilloma and the alimentary tract lesion-derived BPV inoculated calves (91 and 82) while typical rice grain lesions resulted on the teat rice grain lesion-derived BPV inoculated calf (84). At post-mortem, 35 weeks post inoculation, calves 91 and 82 showed extensive fibroblastic meningomas occupying approximately one third of the intracranial left cerebral hemisphere (figure 22 a) and b)). No tumour development occurred in the teat rice grain-derived BPV inoculated calf 84. No lesions developed at any control intracerebral or intradermal inoculation sites.

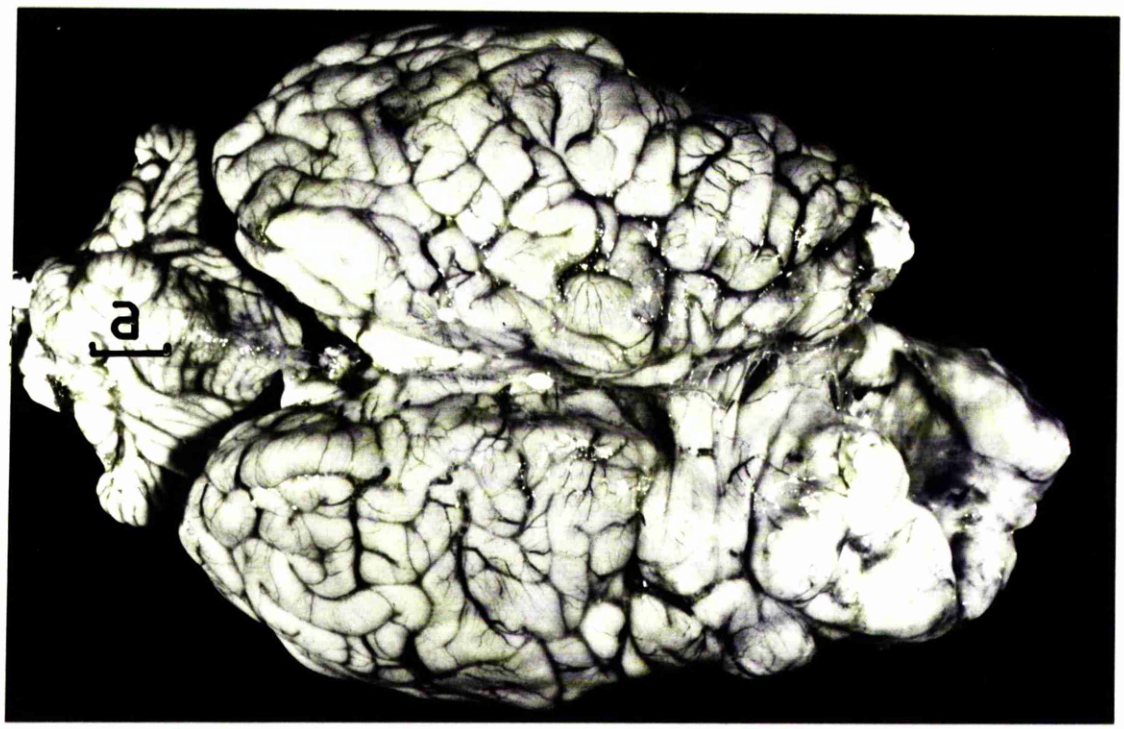


Figure 22a) Macroscopic appearance of BPV-induced meningoma. Calf 91. Bar = 2cm.

← b) Microscopic appearance of BPV-induced meningoma. Calf 91. Bar = 1mm. Stained with H & E.

Calves 45, 81 and 83 which were inoculated with control and BPV transformed or infected foetal bovine skin cells did not develop lesions at either intradermal or intracerebral sites. Haematological examination produced unremarkable results which appear in Appendix C of this thesis. Histological examination of biopsy samples provided results similar to those described in Chapter 3.

Gross examination of the cranial cavity of calves 91 and 82 showed substantial compressive deformation of the left ethmoid turbinates. A normal dura mater covered extensive, multiple smooth round and firm lesions, encapsulated by a grossly deformed left cerebrum and olfactory lobe. Tumours were opaque and white on incision showing a distinctly fibrous texture. Histologically, the tumours showed only rare evidence of invasion of adjacent central nervous tissue and were seen to be continuous with the pia mater. The tumours were composed of relatively avascular, well differentiated fibrous tissue, smaller nodules showing cells which were more fibroblastic in appearance (Figure 22 b)). Histological examination of intracerebral inoculation sites on the right cerebrum of calves 91 and 82 as well as both sites of the other calves showed the presence of carbon surrounded by minor scar tissue. No evidence of an inflammatory response was seen in any intracerebral inoculation site including the meningiomas of calves 91 and 82.

5.4 DISCUSSION

The production of meningiomas experimentally in calves has been reported previously. Gordon and Olson (1968) used twenty, one week old calves and, following intracerebral inoculation, seventeen calves developed fibroblastic neoplasms. Like other papovaviruses (Chapter 1), BPV produces intracranial

fibroblastic neoplasms in the hamster (Robl et al., 1972). This chapter reports two calves which were 4-8 weeks old at inoculation and also developed fibroblastic neoplasms. The macroscopic and microscopic appearance of the tumours were in agreement with those reported earlier.

Gordon and Olson (1968) also reported the transmission of cutaneous fibropapilloma derived BPV to the muzzle, tongue and rumen of calves. Calf 82 reported here was inoculated with BPV extracted from cutaneous fibropapillomas which developed on a calf inoculated with BPV extracted from a pooled sample of alimentary tract papillomas, fibropapillomas and fibromas. (This material was supplied by Professor Jarrett.) This BPV sample was indistinguishable from normal cutaneous fibropapilloma derived BPV in its ability to produce meningomas.

While teat rice grain lesions have been transmitted to the skin of calves (see Chapter 3), neither the naturally occurring lesions nor those transmitted experimentally show evidence of fibrous tissue proliferation. This absence of a mesenchymal response is confirmed by the absence of in vitro transformation of foetal bovine skin cultures as well as the absence of fibrous meningoma production following intracerebral inoculation.

BPV-transformed cell cultures are tumourigenic in nude mice (Chapter 4) and in vitro BPV transformation can be inhibited by antisera from fibropapilloma bearing cattle (Chapter 8). Adsorbtion of such sera with BPV transformed cells; and fibroma, fibropapilloma and BPV induced meningoma tissue markedly reduces the transformation inhibition titre (Chapter 8). The intradermal, submucusal and subcutaneous inoculation of BPV transformed cells has been shown to increase animal resistance to challenge with fibropapilloma derived BPV but not teat papilloma or rice grain lesion derived BPV (Chapter 3). The absence of tumour development following intracerebral inoculation of transformed cells may have been due to the relatively low number (2×10^6) of cells in the inoculum, or high antigenicity of the cells. Another explanation is that the transformed cells are relatively non-oncogenic in the bovine. Considerably more

work will be necessary using larger numbers of animals before these matters can be resolved.

5.5 SUMMARY

Intracerebral inoculation of two calves with fibropapilloma-derived BPV resulted in the production of fibrous meningomas. One of these calves was inoculated with BPV from fibropapillomas which appeared on a calf experimentally inoculated with BPV isolated from a pooled sample of alimentary tract lesions. Three calves inoculated with BPV transformed and untransformed foetal bovine skin cells, failed to produce lesions following intradermal or intracerebral inoculation. One calf inoculated with teat rice grain lesion derived BPV responded with typical rice grain lesions following intradermal inoculation but failed to produce any lesinn following intracerebral inoculation. These findings are discussed in relation to other in vivo and in vitro differences between BPV isolated from morphologically different lesions.

CHAPTER 6: THE EFFECT OF PASTEURISATION ON BOVINE PAPILLOMA VIRUS IN VIVO AND IN VITRO.

6.1 Introduction

In a recent survey (Chapter 2) 36% of all cattle and 48% of parous female cattle were affected with teat papillomatosis. The consistent demonstration of bovine papilloma virus (BPV) in negatively stained preparations of pooled and individual papillomas led to the suggestion that milk from affected cattle could contain virus released by the superficial trauma of machine milking (Chapter 2). Both calves and man may therefore be exposed to a known oncogenic bovine virus when fed milk from affected cows. This chapter reports the in vivo and in vitro effect of pateurisation on different isolates of bovine papilloma virus extracted from three morphologically separable teat papilloma lesion types and two skin fibropapilloma types. The presence of bovine papilloma virus particles in two of six milk samples bought at retail outlets is also recorded.

6.2 Material and Methods

6.2.1 Classification of Lesion Types

Each virus extract was derived from a single case affected with only one lesion type. The three teat papilloma types have been previously described and they were provisionally called frond (F), flat and round (FR) and rice-grain (RG) lesions (Chapter 2). Two cutaneous fibropapillomas are morphologically and epidemiologically separable. The typical cutaneous fibropapilloma described in the literature, involves both epithelial and mesenchymal elements approximately equally. The anogenital fibropapilloma is a cauliflower like lesion, primarily involving mesenchymal elements with secondary epithelial

changes. For a fuller histopathological description of these five lesion types see Chapter 3.

6.2.2 Virus Extraction

Papillomas were removed from cattle and stored separately in PBS/Glycerol at 4°C. One papilloma from each of the five cases was sampled and placed in 10% formol saline and glutaraldehyde fixative for further histopathological and electron microscopic (EM) examination. After histological confirmation of lesion type, papillomas were homogenised using a Silverson blender into a 10% w/v crude suspension in phosphate buffered saline (PBS). This suspension was clarified using an SW27 rotor at 10,000 rpm for 20 minutes in a Beckman L-250 ultracentrifuge. The resulting supernatant was then labelled and stored at -70°C until animals could be infected. Aliquots of each extract were further purified according to methods described earlier (Lancaster et al., 1976) but without the use of detergent or ultrasound to aid virus dispersion.

6.2.3 Electron Microscope Examination

Each crude suspension was negatively stained with phosphotungstic acid (PTA) and examined for the presence of BPV. Purified preparations were similarly examined and virus particle diameter measurements were made as previously described (Chapter 2).

6.2.4 Pasteurisation

One ml of each virus sample was placed in a hot water bath held at 60°C. After the temperature of the sample reached 60°C, thirty minutes was allowed to elapse before the sample was removed and allowed to cool in a refrigerator held at 4°C.

6.2.5 Temperature - Time Inactivation

Five aliquots of the cutaneous fibropapilloma BPV sample were held at 60°C for 30, 60, 120, 180, 240 minutes and subsequently assayed to determine the Log TD_{50}. Six aliquots of the same sample were treated for 30 minutes at 50, 60, 70, 75, 80, 85°C and subsequently assayed to determine the Log TD50. (See Chapter 7 for details of the methodology).

6.2.6 In Vivo Titration

Six major skin sites on each three month-old calf were selected. Each site was prepared by clipping the hair from two 10 x 20 cm areas with at least 10 cm of unclipped hair covered skin left between the two clipped areas.

Each pasteurised and untreated virus isolate was diluted in five 10 fold steps with PBS. Dried, filtered and sterilised activated charcoal was added to each dilution at the rate of 10 mg per ml. Ten 0.1 ml intradermal injections were made for each dilution step into the appropriate designated site. Appendix D summarises the sites inoculated with the different dilutions of both pasteurised and untreated virus isolates. Forty control injections of PBS with 10 mg/ml activated charcoal were made.

Biopsy samples were taken at fortnightly intervals from 10^{-4} virus dilution sites of both pasteurised and untreated isolates as well as control sites. Skin thickness measurements were made of each injection site at the same time and the presence or absence of papilloma was noted. Each biopsy sample was divided into three parts, two parts were fixed in 10% formol saline and glutaraldehyde fixatives for histological and EM examination. The other part was frozen at -70°C for future use either in virus extraction or for cryostat sections.

6.2.7 Milk Samples

Six separate retail outlets were visited in one week and the equivalent of five litres of milk was purchased. Care was taken to ensure that each five litre sample comprised milk from the same dairy and was labelled with the same expiry date or batch number. Samples were labelled A - F and handled separately throughout the experiment.

Each milk sample was diluted 1:4 with distilled water and centrifuged for 10 minutes at 2,000 rpm on a JS 7.5 rotor. The supernatant was removed and the precipitate resuspended in 10 ml of PBS for every 600 ml of original milk sample. Test tubes containing the resuspended precipitate were allowed to stand overnight at $4^{o}C$. The following morning, the supernatant was removed and the uppermost or epithelial component of the cell pellet was carefully resuspended in 0.5 ml of PBS. Epithelial cells were pooled for each sample, washed twice in PBS, pelletted at 2,000 rpm for 10 minutes and resuspended in 1 ml of PBS. Thus each 5 litre milk sample was processed into a single 1 ml epithelial cell suspension in PBS.

These 1 ml suspensions were then subjected to three freeze-thaw cycles (Chapter 2) before being examined negatively stained under the EM for the presence of BPV.

6.3 Results

Table 26 lists the results of pasteurisation on *in vivo* infectivity of virus as measured by the appearance of papillomas and fibromas at the sites of injection. No significant differences were detected between the titres of pasteurised and untreated virus isolates.

Skin thickness measurements detected the presence of an intradermal fibroma at injection sites 2 - 4 weeks before a papilloma appeared in all calves except those

TABLE 26: THE RESULT OF IN VIVO TITRATION OF PASTEURISED AND UNPASTEURISED BPV CONTAINING EXTRACTS ON FIVE CALVES.

CALF NUMBER	68882		68888		68893		68891		68886	
VIRUS SOURCE *	A		B		C		D		E	
VIRUS TREATMENT +	P.	C.	P.	C.	P.	C.	P.	C.	P.	C.
$Log_{10}ID\ 50^{o}$										
- injection sites with papillomas	2.3	2.4	2.3	2.4	2.2	2.3	3.8	3.5	2.4	2.5
- injection sites with fibromas	n.a.	n.a.	n.a.	n.a.	2.5	2.4	3.9	3.8	3.1	2.9

Mean titre $\pm$ S.E. - pasteurised - 2.81 $\pm$ .70
control - 2.78 $\pm$ .57

t value = 0.114; degrees of freed om = 14; significance - n.s.

o $Log_{10}ID50 = Log_{10}$ Infective Dose 50, calculated using method of Reed & Muench.
+ P = pasteurised; C = unpasteurised control
n.a. - not applicable. n.s. - not significant
* See text for description of lesion types corresponding with A - E.

inoculated with TF and TRG. However, no significant differences occurred between maximum mean skin thickness measurements at each dilution site of pasteurised and untreated virus isolates.

When one virus isolate from cutaneous fibropapillomas was held at 60°C for various time intervals, a ten-fold reduction in transformation titre occurred only after four hours at 60°C had elapsed. However, when the same isolate was held for 30 minutes at various temperatures, all detectable transforming activity was lost after 30 minutes at 80°C. Figure 23 illustrates these observations graphically.

The histopathological examination of biopsy samples are reported in greater detail elsewhere (Chapter 3). However, each virus isolate produced experimental lesions identical to the original lesions from which the isolate was first extracted. No differences were detected between lesions produced by pasteurised or untreated aliquots from the same virus isolate. Mean particle diameters as measured on negatively stained preparations have been reported previously (Chapter 2) but are listed on Appendix D for the convenience of the reader.

When freeze-thaw epithelial cell suspensions from milk samples from six separate dairies were examined under the EM using negative staining, two samples yielded particles indistinguishable in size and morphology, from bovine papilloma virus.

6.4 Discussion

Table 12 is a summary of the heat sensitivity of other members of the papova virus group (Chapter 1). Although it can be seen from the above results that BPV is resistant to heat it holds this property in common with other papoviruses. Only one virus, equine cutaneous papillomatosis,

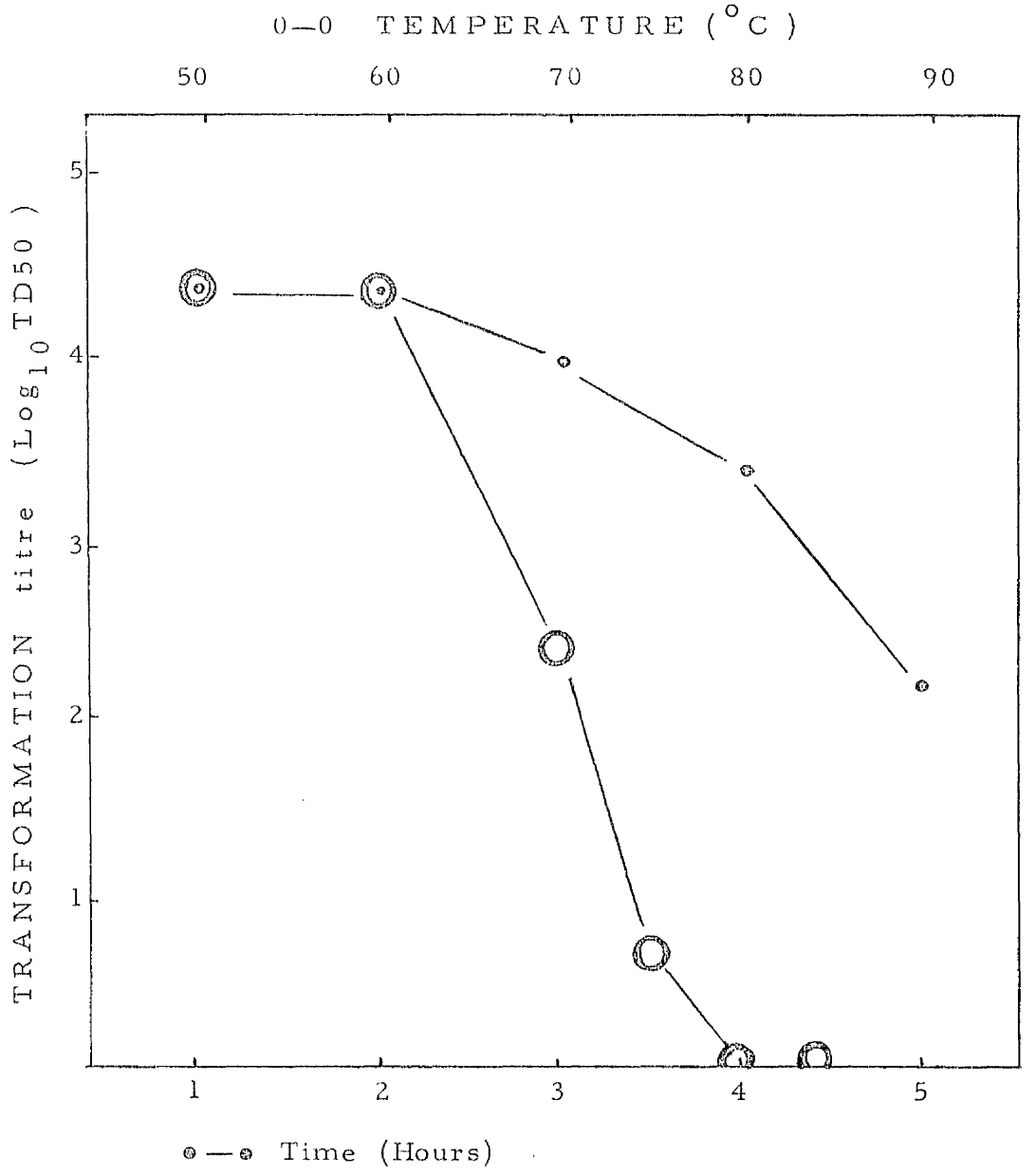


FIGURE 23: The time-temperature inactivation of bovine papilloma virus as measured by in vitro transformation.

- o BPV held for 30 minutes at varying temperatures.
- ⊙ BPV held at 60°C for varying times.

Log_{10}TD50

calculated using methods previously described.

does not survive pasteurisation, that is heating to 60°C for 30 minutes.

The work reported in this paper may tend to underestimate the resistance of BPV in milk to heat inactivation. Samples tested in this report were 10% w/v suspensions of actual papillomas in PBS. Milk containing BPV has a reduced water activity due to the higher content of neutral sugars and so the heat required for complete inactivation may be higher than the experiments indicate. Additionally, the specific gravity of milk approximates that of BPV and so any free BPV in milk is likely to be evenly distributed throughout the sample. When milk from several cows, or from several herds are pooled in a bulk container, it is likely that the whole bulk sample will contain BPV even if only one cow is affected.

These findings suggest that not only calves directly suckling affected cows, but also calves being artificially suckled using pasteurised whole milk or skim milk are likely to be exposed to infectious BPV. These observations are supported by the fact that in the same area where 48% of cows had teat papillomatosis, 19% of all cattle had papillomas of the upper alimentary tract. A similar range of lesion types were reported in the upper alimentary tract as on the teats of cattle (Chapter 2 and Jarrett et al., 1978).

Several reports have suggested that BPV is transmissible to man (Schultz, 1908; Frenz, 1941; Grenier et al., 1976) and this is supported by the observation that persons in certain occupations such as meat handlers are at high risk of developing warts (Perel and Lumpkin, 1976). However, other authors conclude that this is not the case (Rowson and Mahy, 1967). The suggestion that different bovine papilloma viruses occur on bovine teats (Chapters 2, 3 and 5) makes it important to evaluate these viruses in relation to pathologically similar oral lesions already

recognised in man. The naturally occurring bovine teat rice grain lesion yields virus which on transmission produces identical lesions on the skin of calves (Chapter 3) - this lesion is of similar appearance to focal epithelial hyperplasia (Praetorius-Clausen, 1972) in man. The naturally occurring bovine teat frond papilloma yields virus which on transmission produces identical lesions on the skin of calves (Chapter 3) - this lesion is of similar appearance to verruca vulgaris in man (Praetorius-Clausen, 1972). Bovine papilloma virus can be readily demonstrated in milk on retail sale for human consumption, so humans in areas with a high prevalence of bovine teat papillomatosis are being exposed to active virus in pasteurised milk. Further work is necessary to investigate the papilloma viruses of human oral lesions in relation to those causing teat papillomas in cattle.

6.5 Summary

Previous reports of a 48% prevalence of teat papillomatosis among cows in Scotland, led to an investigation of the effects of pasteurisation on bovine papilloma virus isolated from three different teat lesion types and two separable cutaneous fibropapilloma types. No significant differences in transformation titres occurred between pasteurised (60°C for 30 minutes) and untreated virus suspensions *in vitro*. Similarly, *in vivo* titration yielded no significant differences in the number and development of fibromas and papillomas between pasteurised and untreated samples.

A virus suspension of one fibropapilloma type was subjected to varying time-temperature treatments. A tenfold reduction in transformation titre occurred after 4 hours at 60°C and all transformation activity was lost after 30 minutes at 80°C.

Bovine papilloma virus particles were detected in two of six whole milk samples on sale to the public purchased from retail outlets in one week. The importance of these findings are discussed in relation to the high incidence of papillomas in the upper alimentary tract of cattle. A similarity in appearance between lesions on the bovine teat and those reported in the human oral cavity is recorded.

CHAPTER 7 IN VITRO TRANSFORMATION BY BOVINE PAPILLOMA VIRUS.

7.1 Introduction

Bovine papilloma virus (BPV) is a member of the papilloma virus subgroup of the Papovaviridae. BPV has wider cell [illegible] than the other papilloma viruses Intracranial injection of BPV produces meningiomas in calves and fibroblastic intracranial neoplasms in the hamster (Gordon and Olson, 1968; Robl et al., 1972). Subcutaneous injection of BPV produces fibromas in C3H/eB mice and hamsters (Friedman et al., 1963; Cheville, 1966; Robl and Olson, 1968), and intradermal injection of BPV in the horse induces a connective tissue tumour similar to equine sarcoid, naturally occurring cases of which have been shown to contain BPV virus-specific DNA sequences (Olson and Cook, 1951; Lancaster et al., 1977).

Reports during the early 1960's describe transformation in vitro by BPV. Foetal bovine conjunctiva and kidney cell lines as well as secondary cultures of foetal bovine heart transformed after infection with BPV (Black et al., 1963). BPV also transformed embryonic primary cell cultures from C3H/eB, C57/BL and BALB/c mice (Thomas et al., 1964) as well as foetal bovine skin primary cultures (Thomas et al., 1963). Since that time, no further reports concerning the use of BPV induced transformation as an assay system have been published. Unlike other members of the Papovaviridae, no reliable in vitro method has been reported for the growth or the quantification of BPV. Physical particle counting using the electron microscope or biochemical protein estimations of particle numbers both depend on a long, potentally damaging and wasteful procedure to purify BPV from in vivo lesions. Even so, they give no

indication of the biological activity of BPV in any given preparation.

This paper reports the search for suitable foetal bovine cell cultures for use in a transformation assay and the development and characterisation of a quantal transformation assay for BPV.

7.2 Materials and Methods

7.2.1 Primary Cell Cultures

Table 27 lists the various tissues collected from bovine foetuses at differing stages of gestation as measured by foetal length (nuchal crest to tail butt). Approximately 0.5 to 1 gram of foetal tissue, collected under sterile conditions, was rinsed three times in Basal Eagles medium (BME). 10 ml of BME was added to the tissue and the mixture treated in a Stomacher (A.J.Steward, Blackfriars Road, London) for 1-5 minutes depending on the type of tissue. The resultant cell suspension was then evenly divided into four 8oz glass medical flasks already containing 20 ml BME with 20% added foetal bovine serum. 7-10 days after seeding, bottles were examined for evidence of cell growth. Those which showed growth without evidence of contamination were passaged at a 1:2 ratio. This procedure was repeated at weekly intervals using BME with 15% foetal bovine serum until the cultures stopped growing.

7.2.2 Skin Cells

Three collection methods were used to obtain material in attempts to establish foetal bovine skin cultures. (1) Pieces of foetal bovine skin (3-5 mm diameter) were treated in the Stomacher as described above; (2) 2-3 pieces of foetal bovine skin were placed in 8oz medical flasks, epidermis uppermost, in 20 ml BME with 20% added foetal

bovine serum; (3) an area 4 cm x 2 cm of the skin of a bovine foetus was lightly scraped with a sterile scalpel blade and the scrapings were placed in an 8 oz medical flask in 20 ml of BME with 20% foetal bovine serum.

7.2.3 Meningeal Cells

The vascular meninges (pia mater and arachnoidea) were separated from the fibrous meninges (dura mater). Both were treated in the Stomacher before seeding on to 8oz medical flasks. In bovine foetuses of length > 40 cm, the meninges were unavoidably contaminated with a considerable amount of brain tissue. In smaller foetuses, it was possible to separate the different components.

7.2.4 Virus

A 10% w/v homogenate of papilloma tissue was made in phosphate buffered saline (PBS) using a Silverson (Silverson Machines Ltd., Waterside, Chesham, England) blender. This was centrifuged at 2,000g for 20 minutes at $4^{o}C$ in a Beckman L2 - 65 ultra centrifuge using a SW27 rotor. Purified virus was prepared according to methods described earlier (Lancaster et al., 1976) but without the use of detergent or ultrasonic dispersion of pellets.

7.2.5 Infection Of Cells In Suspension

A confluent monolayer of each secondary cell culture was treated with trypsin/versene and after washing the suspension of cells in BME + 10% foetal bovine serum, the cells were seeded onto each well of a 24 x 1 cm Falcon Multiwell (Becton & Dickinson Ltd., Brindlay 74, Runcorn, Cheshire, UK) plate at the rate of $3 - 5 \times 10^{4}$ cells per well in 0.9 ml of BME with 15% foetal bovine

serum. Within 10 minutes, 0.1 ml of virus suspension at serial 10 - fold dilutions was inoculated into each well. Each dilution of virus was inoculated into 4 wells, thus the resulting multiwell plate contained 4 replicate 10-fold serial virus dilutions of 10^2, 10^3, 10^4, 10^5, 10^6 plus 4 control wells which were inoculated with .1 ml 10% crude suspension of normal skin taken from the same animal as the virus preparation. For assay purposes, the Log_{10} 50% transforming dose (TD50) determined using 10-fold step dilution, was more accurately confirmed using replicate two-fold dilution steps around the transformation end point.

7.2.6 Infection of Cells As Monolayer

Multiwell plates were seeded with 3-5 x 10^3 cells in .9 ml BME + 15% foetal bovine serum per well and placed in a humidified incubator with a 5% CO_2 in air atmosphere at 37°C overnight. The following morning 0.1 ml control and virus containing suspensions (as described above) were inoculated into the wells.

7.2.7 Examination Of Plates

Medium was removed from each plate, the cells fixed for 30 minutes in methanol, rinsed with water and then stained for 20 minutes using Giemsa. Plates were rinsed again in water and allowed to dry upended on tissue paper. For each plate gross examination (figure 24) was followed by examination on a Leitz inverted light microscope.

7.2.8 Calculation Of Log TD_{50}

The Karber modification of the Reed and Meunch method was used to determine the end point titration of various virus preparations. To estimate the accuracy and reproducibility of transformation, three cell cultures were

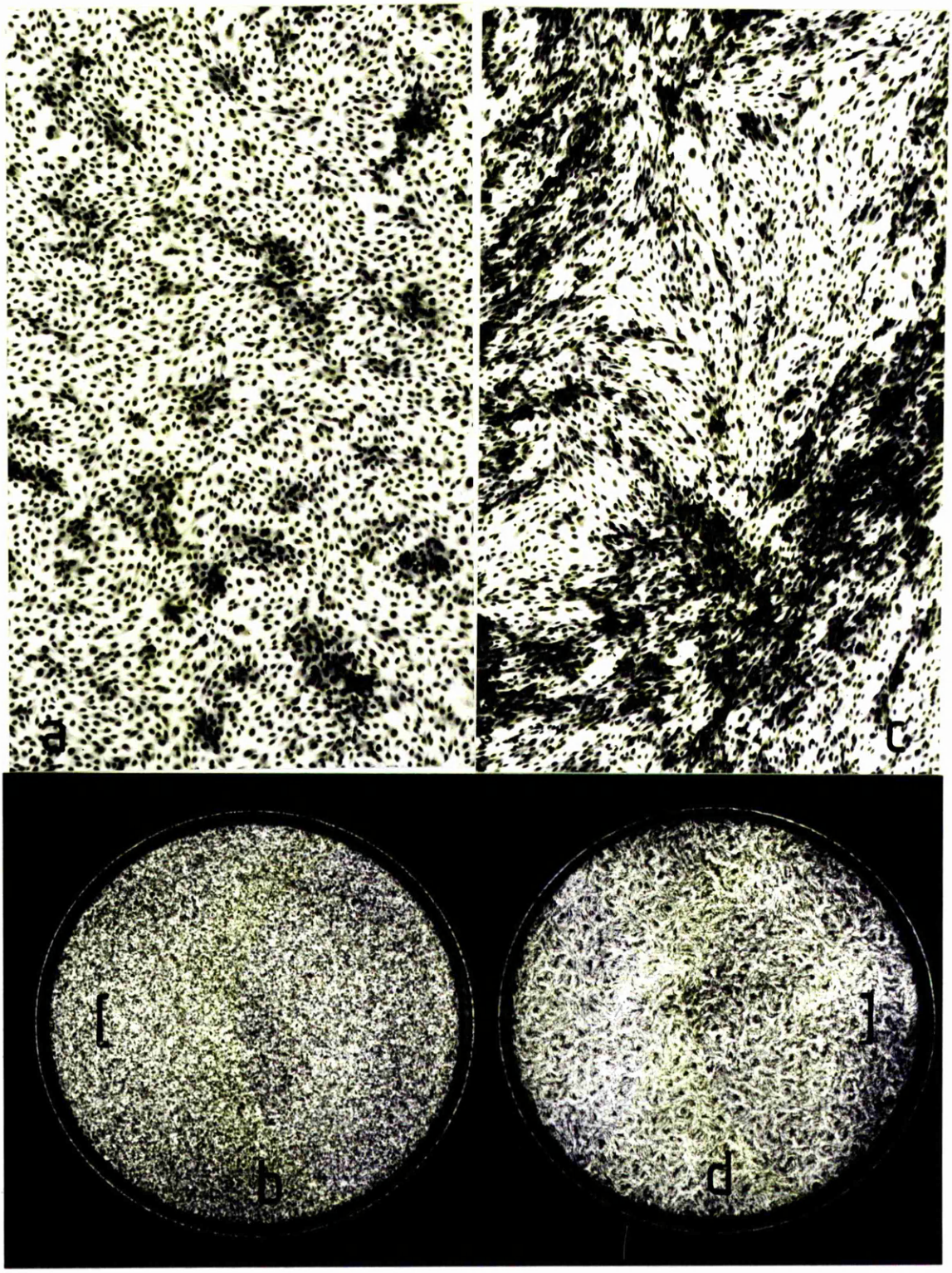


FIGURE 24 BPV transformation of bovine skin cultures. a. and b. low power and macroscopic appearance of control cultures. c. and d. low power and macroscopic appearance of BPV transformed cultures. Giemsa stain. Bar = 2 cm.

infected with 10 separate aliquots of BPV made from (1) a single case papilloma pool; (2) 10 separate papillomas from the same animal.

7.2.9 Characterisation Of Transforming Cell Cultures

Each cell culture which showed transformation was assayed to determine the optimal time for examination. Ten multiwell plates plus cells were prepared, infected in suspension with serial BPV dilutions and stained at 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days post infection. The cells were not passaged, however the medium was replaced at weekly intervals.

7.2.10 Adsorption Time

A foetal bovine skin culture was used to determine the adsorption time for BPV both after infection in suspension and as a monolayer.

Following inoculation with BPV, the medium was removed at intervals as shown on figure 25; the wells were rinsed twice with PBS and then 1 ml of fresh BME + 15% foetal bovine serum placed into each well. The plates were examined at the optimal time for transformation determined earlier.

7.2.11 Generation Interval

Paired transformed and control cultures were examined at regular passage intervals to compare growth characteristics. A suspension of cells was made using trypsin/versene, cell clumps were disassociated by vigorous pipetting prior to counting in a haem ocytometer. Viability was estimated using the trypan blue exclusion method. Generation intervals during the log phase of growth were calculated from growth curves plotted for each culture. Saturation densities were also determined

for each culture.

7.2.12 Estimation Of Cell Longevity

Paired uninfected and transformed cell cultures were passaged at weekly intervals at a ratio of 1:2 until cell growth ceased. Four replicates of each culture were treated identically for this work.

7.2.13 Agar Suspension Culture

Attempts were made to grow transformed cells in agar suspension cultures using the method described by MacPherson and Montagnier (1964).

7.2.14 Haemagglutination

The haemagglutinating activity of BPV preparations was determined using mouse erythrocytes according to the technique described by Favre et al. (1974). Six replicate titrations using two-fold dilution steps were used to determine the Log_{10}HAD 50.

7.3 Results

Table 27 shows foetal length, the tissues collected, cultures which grew beyond the third passage and those which showed transformation after infection with BPV. Cell cultures from foetal bovine palate, conjunctiva and vascular meninges were consistently transformed by BPV. Only two cell cultures from foetal bovine skin transformed. These were two cultures established using the epidermal scraping method - cultures from the same foetuses established using whole skin pieces or 'stomached' skin pieces were more fibroblastic in appearance and did not transform after infection with BPV. No other foetal bovine skin cultures established by any of the three methods transformed with BPV. It should be noted that the foetuses from which the transformation sensitive skin cultures were

TABLE 27: FOETAL BOVINE TISSUES COLLECTED, CELL GROWTH PAST 3rd PASSAGE AND TRANSFORMATION SENSITIVITY TO BPV OF FOETAL BOVINE CULTURES

Foetal Number	1	2	3	4	5	6	7	8	9	10	11	12	13	Transformable Cultures/ cultures Established
Foetal Length (cm)	12	20	21	25	16	11	15	22	50	55	60	56	24	
Tissue collected														
amnion	-	-	-											
skin	-	*	*	*	*	*	-	*	*	*	*√	*√	*	2/11
palate	-	*√	-	-	-	-	-	-	*√	*√	-	-	-	3/3
conjunctiva	*√	-	*√	-	-	-	-	*√	*√	-	*√	*√	-	6/6
oesophagus	-	-	*											0/1
stomach	-	-	-											
intestine	-	-	-											
trachea	-	-	-											
lung	*	-	-											0/1
thymus	*	-	*											0/2
liver	-	-	-											
spleen	-	-	-											
kidney	*	-	*											0/2
brain	-	-	-											
teat	-	*	-											0/1
bladder	-	-	-											
vascular meninges	*√	*√	-	*√	*√	-	*√	*√	*√	*√	-	-	*√	9/9
prepuce	-		*											0/1
testis	-		-				*	*	*					0/3
fibrous meninges				*	-	*	-	*	*					0/4

* = growth > 3rd passage √ = transforms with BPV - = no growth > 3rd passage
blank = not collected.

isolated were both near-term, showing approximately 1 cm hair length.

The features of transformation were similar in all cultures. Cultures first showed morphological changes between 3-5 days post infection, with the concurrent appearance of 'floater' cells in the medium and long spindle shaped cells showing disorganised growth patterns. The spindle cells overgrew the other cells and formed into large bundles or cords of rapidly growing cells. At the junction of these cords the spindle cells crossed over each other to form the typical transformation foci associated with cell transformation by other members of the papova-virus group. The medium in infected wells showed considerable acidification when compared with control wells. Between days 5 - 7 these cords of cells had overgrown to the extent that they were visible macroscopically as "wisps" (Figure 27).

Comparison between BPV infection of cells in suspension and as a monolayer consistently showed the appearance of wisps to be delayed by 3 - 4 days in the monolayer infected cultures. The Log TD 50 did not differ significantly between the two infection methods. The delay in appearance of wisps could be ascribed to differences in adsorption time (Figure 25).

Figure 25 shows the time necessary for complete virus adsorption to take place to produce maximum Log TD 50. Complete adsorption of BPV takes 3 - 4 days when cells are infected in suspension and up to 7 days when cells are infected as a monolayer.

Repeated passaging of paired transformed and control cultures showed a consistent increase in the longevity of transformed cultures when compared with controls. Table 28 shows these differences for four cultures thus far observed in this laboratory. In each

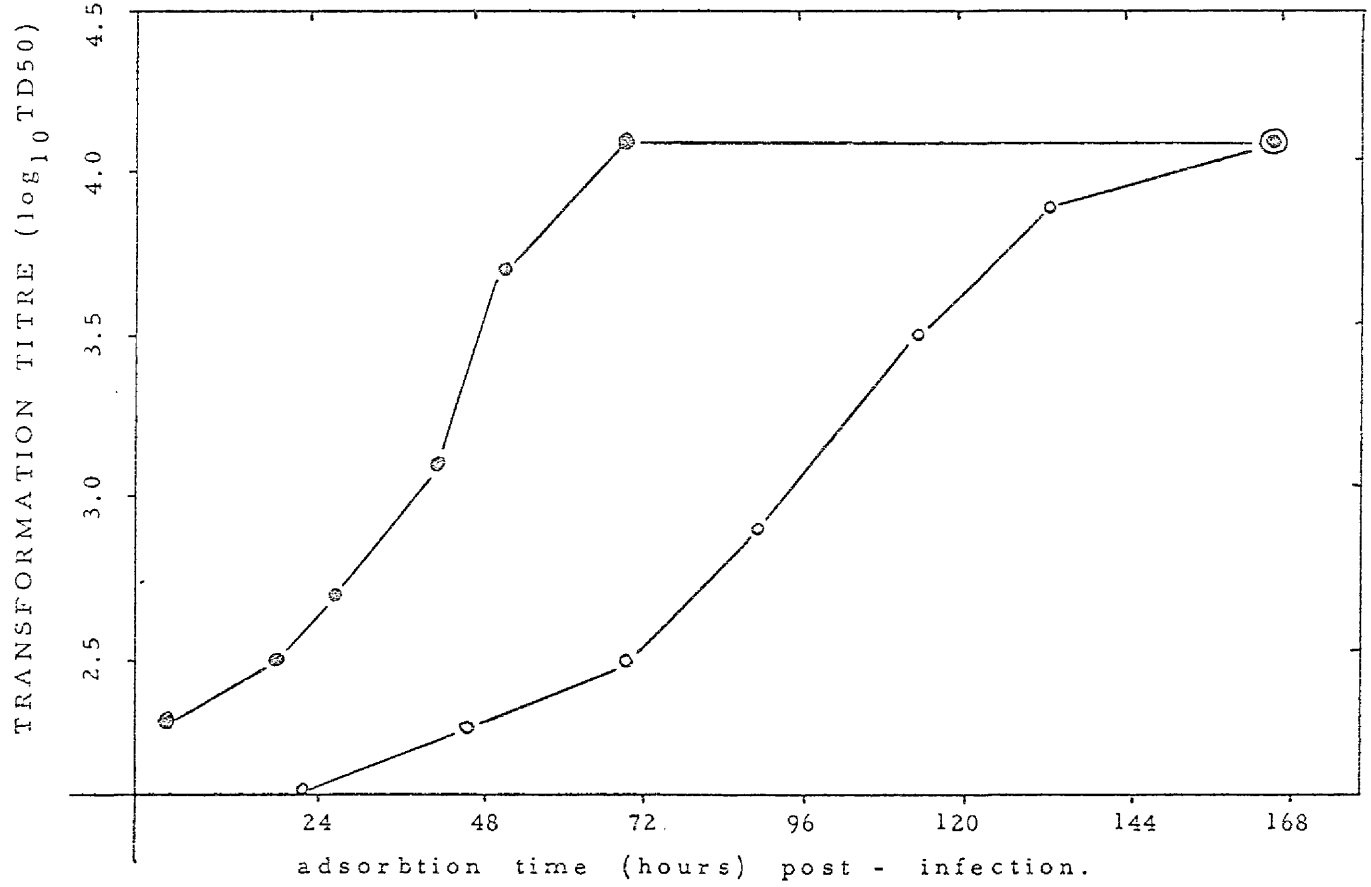


FIGURE 25: Effect of different adsorbtion times on transformation titre of bovine papilloma virus infected foetal bovine skin cultures. ⊖ Suspension infected, assay read at 7 days; o monolayer infected, assay read at 12 days.

TABLE 28: LONGEVITY OF PAIRED TRANSFORMED AND CONTROL CULTURES.

CULTURES *	TRANSFORMED		CONTROL	
	Total No. Passages	Time (months)	Total No. Passages	Time (months)
2 Meninges	32	8	9	2
3 Conjunctiva	20	5	8	2
5 Meninges	27	6	11	3
(8A)Meninges	25	6	8	2

* four replicate bottles used for each cell culture.

TABLE 29: THE RESULT OF TRANSFORMATION ASSAY OF TEN SEPARATELY PROCESSED PAPILLOMAS FROM A SINGLE CASE (65819) ON THREE DIFFERENT FOETAL BOVINE CULTURES.

	Transformation Assay Result * $Log_{10} TD_{50}$			Haemagglutination +
Cell Culture	11 Skin	12 Skin	12 Conjunctiva	$Log_{10} HAD_{50}$
Mean titre ± S.E.	3.44 ± 0.32	3.58 ± 0.10	3.62 ± 0.17	3.08 ± 0.35
t-value	$11S/_{12S}$ 1.306	$12S/_{12C}$ 0.692	$12C/_{11S}$ 1.594	
degrees of freedom	18	18	18	
Significance	n.s.	n.s.	n.s.	

* determined using 4 replicate 2 step serial titration for each sample.

\+ determined using 6 replicate 2 step serial titration for each sample.

n.s. not significant.

case the macroscopic wisps of the transformed cultures persisted throughout their lifetime.

No significant differences occurred between the generation intervals or saturation densities of transformed and control cultures after inoculation and through to cell death, several months later. However, figure 26 illustrates differences in growth rate after BPV infection, with an initial depression (day 0 - 3) in growth rate, a large amount of cell death, and then a potentiation of cell growth so that monolayers observed at days 8 - 12 showed similar cell numbers to controls.

Transformed cells did not form colonies during a four week observation period after suspension in 0.3% agar. Three different cell cultures, each transformed by different BPV isolates, were tested and in no case was colony formation observed.

When ten papillomas from a single case (65819) were separately processed into individual 10% crude suspensions and then assayed on three different cell cultures, there were no significant differences between the mean Log TD50 of each culture (Table 29) using 'Student's t-test'. When ten separate aliquots of a 10% crude suspension of another case (66702) were assayed on the same three cell cultures, no significant differences were found between the mean Log TD50 of each culture (Table 30). Appendix E contains the detailed results of work reported here. It should however be noted, that in all cases the mean titres of separately processed papillomas had a higher standard error when compared with mean titres of separate aliquots of a single pooled papilloma sample. In both experiments, the HA titres were lower than the transformation titre, and the standard errors were higher indicating the lower precision of the HA assay compared with the transformation assay.

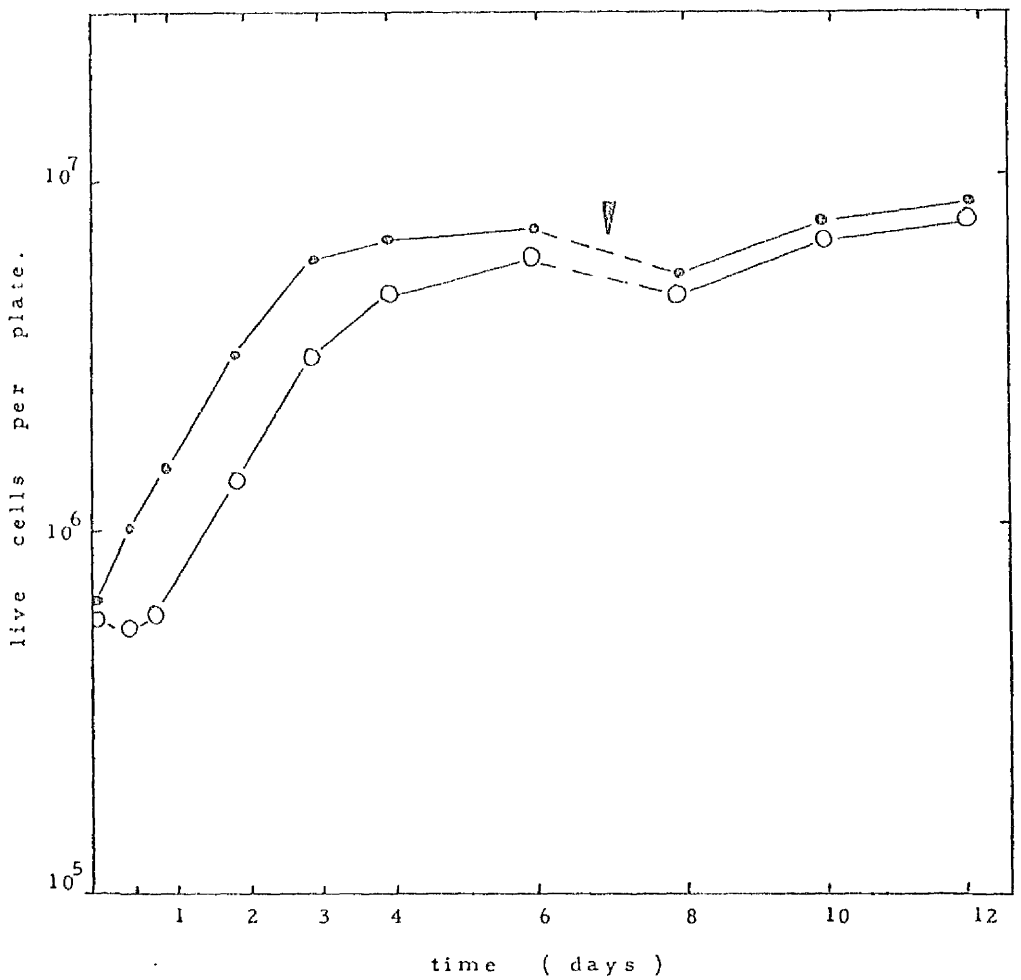


FIGURE 26: Effect of bovine papilloma virus on growth curve of a foetal bovine skin culture.

o culture infected with BPV at time o in suspension

o paired control culture infected with normal skin extract at time o in suspension.

▽ medium change.

Determination of the optimal time for reading assay plates showed that there was considerable variation between cell cultures. In each cell culture there was a 2 day interval between the time when microscopic evidence for BPV transformation was present without gross wisp formation and when cultures were so over-confluent that gross examination for wisps was difficult. During this 2 day interval both microscopic and macroscopic evidence for transformation was clearly evident, and this is the period used to read assay plates. Table 31 illustrates the variability between cell cultures, showing the time of first appearance of wisps in monolayer infected as well as suspension infected cultures.

BPV extracted from cutaneous fibropapilloma, anogenital fibropapilloma, alimentary fibropapilloma and teat fibropapilloma of cattle have all induced transformation in the four most studied cultures (11 and 12 skin; 2 meninges and 12 conjunctiva). Virus extracted from canine skin papilloma has not induced transformation in any of these four cultures, similarly, teat papilloma and rice grain lesion derived BPV has not induced transformation in the abovementioned four cultures.

7.4 Discussion

The results presented in table 27 show that cultures derived from foetal bovine meninges, conjunctiva, and palate, consistently transformed after infection with BPV. Black et al., (1963) found that BPV transformed bovine kidney and foetal bovine conjunctiva (DBC) cell lines, as well as secondary cultures of foetal bovine heart. Primary bovine kidney cultures however, did not transform nor did the two foetal bovine kidney cultures reported in this paper. Thomas et al., (1963 and 1964) reported BPV induced transformation of foetal bovine skin cells and primary

TABLE 30: THE RESULT OF TRANSFORMATION ASSAY OF TEN ALIQUOTS OF POOLED PAPILLOMAS FROM A SINGLE CASE (66702) ON THREE DIFFERENT FOETAL BOVINE CULTURES.

	Transformation Assay Result* Log_{10}TD50			Haemagg-lutination+
Cell Culture	11 Skin	12 Skin	12 Conjunctiva	Log_{10} HAD50
Mean Titre ± S.E.	4.24 0.06	4.22 0.07	4.21 0.06	2.53 0.34
t-valve	$^{11S}/_{12S}$ 0.49	$^{12S}/_{12C}$ 0.45	$^{12C}/_{11S}$ 0.99	
degrees of freedom	18	18	18	
significance	n.s.	n.s.	n.s.	

* determined using 4 replicate 2 step serial titration of each aliquot.

\+ determined using 6 replicate 2 step serial titration of each aliquot.

n.s not significant

TABLE 31: TIME OF FIRST APPEARANCE OF TRANSFORMATION WISPS IN SUSPENSION AND MONOLAYER INFECTED CULTURES.

	Time Of First Appearance Of Wisps(days)	
CELL CULTURE	SUSPENSION INFECTED	MONOLAYER INFECTED
2 Meninges	10	14
5 Meninges	9	13
10 Meninges	7	11
11 Skin	8	12
12 Skin	8	12
12 Conjunctiva	10	14

cultures derived from foetal bovine skin pieces. This chapter reports only two foetal bovine skin cultures which transform, and these were derived from epidermal scrapings of two near term foetuses. Parallel cultures from the same foetuses, using whole or dispersed skin pieces, failed to transform, as did cultures from the skin of younger and smaller foetuses. Although BPV induced tumours are known to occur in bovine meninges (Gordon & Olson, 1968) and palate (Jarrett et al., 1978) in vivo, transformation by BPV of cultures from these tissues in the foetus, has not been previously recorded.

The microscopic appearance of BPV induced transformation has already been described (Black et al., 1963; Thomas et al., 1963 & 1964) however the development of gross wisps when transformed cultures reach overconfluence, has not been reported previously. It is these wisps which provide a sharply defined end point, making a quantal transformation assay more attractive and easy to perform, when compared with existing BPV assay systems. In over 200 assays conducted, a partly transformed well has never been observed; the presence or absence of wisps appears to be an all or nothing phenomenon. In contrast, the pleomorphic nature of some cultures makes examination for microscopic foci very difficult, with the result that end points are obscure.

Transformed cultures showed a 2.5 - 3-fold increase in lifespan without loss of macroscopic wisps, compared with paired control cultures. Earlier papers have reported increased longevity before the complete lifespan of transformed cultures had been determined (Thomas et al., 1963 & 1964).

Suspension infected cultures consistently showed transformation wisps 3 - 4 days earlier than monolayer infected cultures in the total time required for transformation wisps to appear. These intervals contrast with the

period of 45 days reported by (Thomas et al., 1963) for foetal bovine skin, but are similar to the 6 - 10 days seen in mouse cells (Thomas et al., 1964) and the 3 - 10 days observed with bovine conjunctiva (DBC) cells. DBC suspension infected cultures transformed 7 days earlier than those infected as a monolayer (Black et al., 1963).

The difference between adsorption times of the two infection methods, parallels the delay in appearance of transformation wisps in monolayer infected cultures. Previous reports of low transformation titres may have been due to incomplete BPV adsorption.

Transformed cells did not form colonies in agar suspension, and there were no significant differences in the generation intervals or saturation density of paired transformed and control cultures.

Mean titres calculated from repeated assays on three different cultures showed no significant differences. This occurred both using ten separately processed papillomas from a single case, and ten aliquots of a single papilloma case. These results indicate that the transformation assay is reproducible on cultures derived from different tissues of the same foetus, and from the same tissues of different foetuses.

The use of 1 cm diameter multiwell plates has made the assay procedure both easier to perform and has allowed considerable economies in the use of cells and reagents. Four separate multiwell plates can be prepared using one confluent 8oz medical flask of primary culture. Haemagglutination titres in this work varied between 0.5 and 2 $\log_{10}$ dilutions less than equivalent transformation titres. Transformation titres in this laboratory have varied between 0 and 6.2 indicating greater sensitivity compared with the haemagglutination. The comparative results indicate that the transformation assay

is measuring a different parameter to the haemagglutination assay.

The effects of inoculation of BPV transformed cell cultures into cattle and nude mice has been reported in Chapters 3, 4 and 5 of this thesis. Only three fibropapilloma extracts have not caused in vitro transformation, compared with over fifty which have. These non-transforming isolates are discussed in greater detail in Chapter 8.

7.5 Summary

Forty-four cultures grew past the third passage from twenty different tissues sampled from thirteen bovine foetuses of differing sizes. Twenty cultures transformed after infection with bovine papilloma virus. All foetal bovine palate, conjunctiva and vascular meningeal cultures transformed however only epidermal scraping of skin from near-term foetuses yielded transformable skin cultures. Transformed cultures showed heritable microscopic and macroscopic changes which persisted throughout their lifespan. Although no differences were detected in generation interval or saturation density, transformed cultures survived 2.5 - 3 times as long as paired control cultures. Transformed cells did not produce colonies when grown in agar suspension. Unlike previously reported microscopic foci, macroscopic wisps, formed when BPV infected cultures reach overconfluence, provided a clearly defined end point for a quantal transformation assay. No significant differences resulted between mean titres of the same viral extract on two skin cultures of different foetuses or a skin and a conjunctival culture from the same foetus. BPV extracts from bovine teat, skin and alimentary fibropapillomas transformed all four cultures tested, however extracts from canine skin papilloma, teat papilloma and rice grain lesions did not.

A consistent difference of 4 days occurred in both the BPV adsorption time and the time taken for transformation wisps to appear when cells infected in suspension were compared with monolayer infected cultures. The use of 1 cm diameter multiwell plates resulted in an easier and more economical assay procedure. The quantal transformation assay reported in this chapter has a greater sensitivity and precision but may be measuring a different BPV parameter than BPV haemagglutination.

Chapter 8 Inhibition of in vitro transformation by bovine papilloma virus

8.1 Introduction

Fibropapilloma derived BPV has been shown to produce tumours of both epithelial and mesenchymal tissues in a wide range of anatomical locations in cattle (Gordon and Olson, 1968; Chapters 2, 3, 5 of this thesis). In vitro transformation of foetal bovine skin, palate, conjunctiva and meningeal cultures has been described earlier (Chapter 7 and references therein).

Anti-viral antibodies have been found in fibropapillomas (Smithies and Olson, 1961) using immuno-fluorescence. They may be quantitated using immunodiffusion (Koller et al., 1974) and haemagglutination inhibition (Favre et al., 1974). No correlation was found between fibroma regression and anti-V antibody by Barthold and Olson (1974a). The fibroma cells of BPV induced fibropapillomas lack V-antigens detectable by IF and EM (Smithies and Olson, 1961; Tajuna et al., 1968; Robl and Olson, 1968) but possess a surface antigen detected by indirect IF on live unfixed BPV induced fibroma cells grown in vivo and in vitro (Barthold and Olson, 1974b). Serially sampled fibropapillomas show considerable histopathological evidence of a major role played by CMI in the regression of at least the fibroma component of bovine fibropapillomas (Barthold and Olson, 1974b; Chapter 3 of this thesis).

The investigation reported in this chapter was motivated by the need to show convincingly that the BPV in vitro transformation was indeed BPV specific. Furthermore, despite repeated attempts using a variety of techniques reported in the literature (Koller et al., 1974; Almeida and Goffe, 1965 and others - see Chapter 1.5), double immunodiffusion of a variety of virus isolates, antigen preparatory techniques and sera from cattle with regressing fibropapillomas failed to produce distinct precipitin lines. This chapter reports experimental work on the inhibition of in vitro viral transformation.

8.2 Materials and Methods

8.2.1. Virus, Cells, Sera and Complement.

The isolation and growth of transformation sensitive foetal bovine cell cultures has been described earlier (Chapter 7). One standard 10% w/v virus suspension was used throughout this work (66702 - see Chapter 7). Virus was stored in aliquots at -70°C until use.

Sera were collected from a number of experimental calves, experimentally inoculated with different virus isolates (See Chapters 3 and 5). The sera were heat inactivated at 60°C for 30 minutes before being held at -20°C until further use.

A standard source of complement was used. Two one week old lambs were exsanguinated and serum aliquots of 5ml were stored at -70°C till use.

8.2.2 Assay Procedure

24 well Falcon multiwell tissue culture plates were used. Figure 27 illustrates a typical test plate. Wells A1 and B1 received control cells alone and control cells plus antiserum plus complement respectively. Wells C1 and D1 received cells plus virus plus complement and cells plus virus respectively. All wells in rows 2-6 inclusively received cells plus virus at 10^{-2} dilution (equivalent to approximately 100 transforming units per well) plus complement. Test sera were diluted ten-fold steps using Eagles medium without added foetal bovine serum. Rows 2, 3, 4, 5 and 6 received undiluted, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of test sera. Complement was added as indicated above to an on plate concentration of 5% by volume. Each 1ml well thus contained ~0.8ml cells, medium with 15% added FBS, complement; 0.1ml test serum at appropriate dilutions; and o.1ml of virus at fixed dilution of 10^{-3}.

Plates were placed in a 5% CO_2, humidified incubator held at 37°C. Three days later, the medium was removed from each well and replaced with 1ml BME with 15% added FBS. Plates were stained with Giemsa and examined for evidence of transformation (see Chapter 7). The 50% transformation inhibition dose (Log_{10} TID50) was calculated using the method of Reed and Munch.

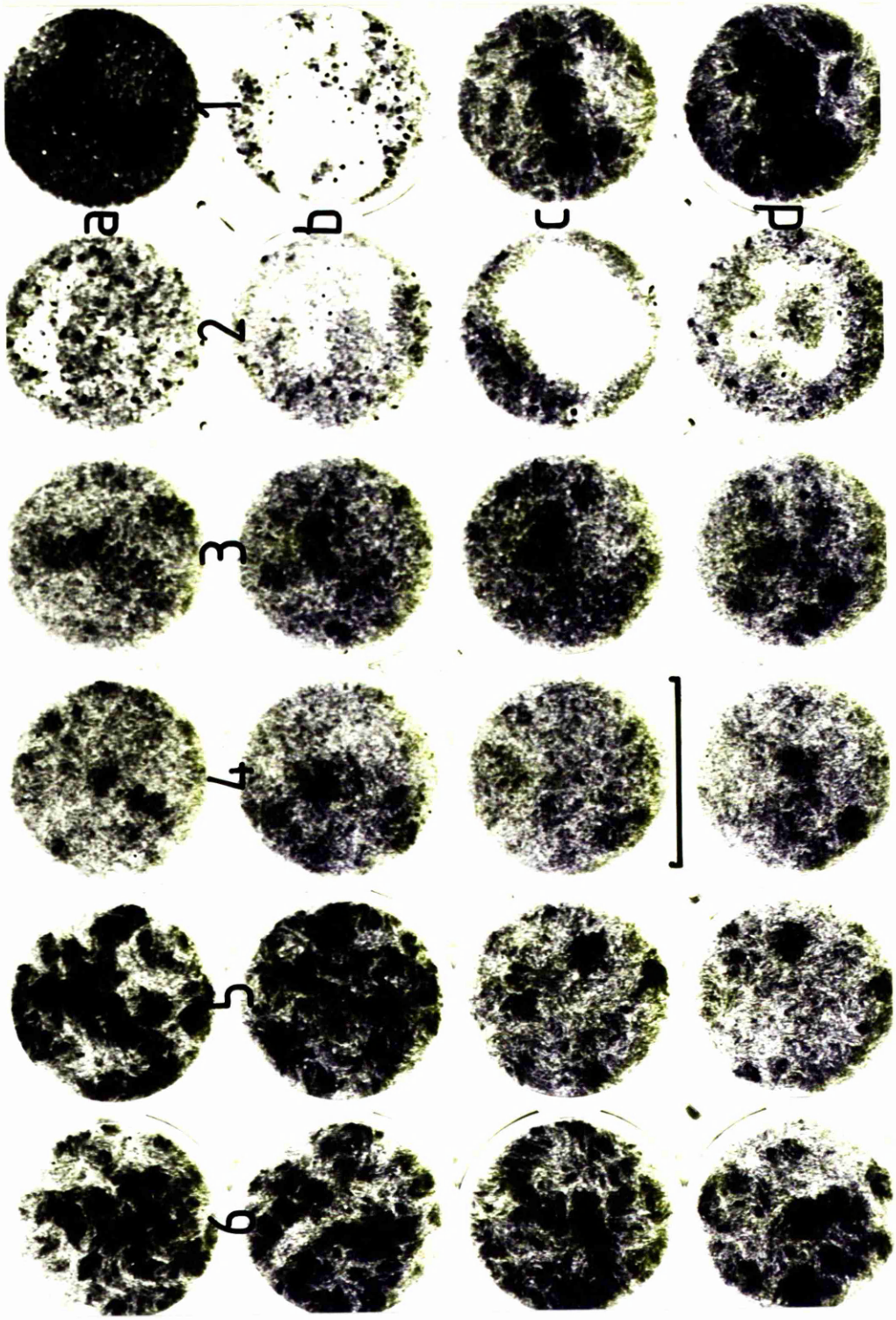


Figure 27 BPV in vitro transformation inhibition assay test plate. Contents of wells were: A1 - cells alone; B1 - cells plus antiserum plus complement; C1 - cells plus virus plus complement; D1 - cells plus virus; Row 2, 3, 4, 5 and 6 received neat 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of antiserum, 5% complement and 10^{-2} BPV respectively. The Log_{10} TID50 of this serum (68888 W18) was 2.5. Stained with Giemsa. Bar = 1cm.

8.2.3 Varying complement and virus concentrations

Preliminary experiments had shown that the transformation inhibitory (TI) effect was absent when both the test sera and FBS in the medium were heat inactivated before use. The TI effect could be restored using an alternative source of complement (initially guinea pig serum, but subsequently newborn lamb serum).

Varying concentrations of virus were used with standard concentrations of complement and test serum, to investigate their effect on the serum TID 50. Additionally, varying concentrations of complement were used with standard concentrations of virus and test serum to investigate their effect on the serum TID 50

8.2.4 The effect of high titre sera on previously transformed cell cultures

A cell culture transformed with BPV (66702) was used to investigate whether high TID 50 sera inhibited the phenotypic expression of transformation reported earlier (Chapter 7). Test procedure was as described earlier (8.2.2) with the difference that the control wells 1A, 1B, 1C and 1D all contained transformed cells.

8.2.5 Adsorbtion of high titre sera with various BPV related suspensions.

High titre serum 68893 week 34 was adsorbed with an equal volume of various BPV-related suspensions listed in table 32. Following thorough agitation, the mixtures were placed in a water bath held at 37°C for 30 minutes before being held at 4°C overnight. The following morning the mixtures were centrifuged for 30 minutes at 2,000 rpm on a bench top

TABLE 32 The effect on transformation inhibition titre of prior serum adsorbtion with various BPV related suspensions (serum 68888 W24)
(* these are significantly ($P < 0.01$) different from the other titres)

	Adsorbant	Log_{10} TID 50
Controls	none	3.5
	normal bovine skin	3.5
	normal foetal tissue	3.0
	normal foetal tissue culture cells (10^7)	3.25
CsCl purified virus	teat fibropapilloma	3.5
	teat rice grain lesion	3.5
	dog skin papilloma	3.5
	teat papilloma	3.5
	anogenital fibropapilloma	3.25
	cutaneous fibropapilloma	1.75*
clarified 10% w/v suspensions	teat fibropapilloma	3.5
	teat rice grain lesion	3.75
	dog skin papilloma	3.5
	teat papilloma	3.5
	anogenital fibropapilloma	3.0
	cutaneous fibropapilloma	3.25
tissue pieces or cells	cutaneous fibropapilloma	1.5*
	cutaneous fibroma	1.75*
	BPV transformed cells (10^7)	1.0*
	BPV meningoma	1.0*
	teat papilloma	3.0
	rice grain lesion	3.5

centrifuge.

Each supernatant was then decanted and tested to determine the TID 50 as described above (8.2.2). To compensate for the 50% dilution of the sera during adsorbtion, 0.2ml instead of 0.1ml was added per test well.

8.2.6 Investigation of effect of non-transforming isolates

Chapter 7 reports that three of over 50 fibropapilloma isolates failed to transform cultures in vitro. Nevertheless, these isolates were shown to induce morphological changes in vitro which were reproducible and titred out to low titres of between $10^{-1.5}$ and $10^{-2.3}$. One of these isolates was included both in the adsorbtion experiments outlined above (8.2.5). In addition, isolates of dog papilloma, teat papilloma and rice grain lesions were included in the adsorbtion experiments.

A two stage assay was developed to detect any additive or interference effects of nontransforming virus isolates on the titre of transforming isolates. In this interference assay the first stage involved preparing a typical transformation assay (described in section 7.2) using a nontransforming isolate. Four days later, cells on each well were suspended with trypsin/versene and divided over two fresh multiwell dishes - each well which had received a particular nontransforming virus dilution retaining its position on the new plates. Both new plates were then inoculated with a transforming virus isolate of known titre in a manner identical to a standard transformation assay (7.2), except that each treated well received the equivalent of ten transforming units of the second virus. Further experiments involved the simultaneous infection of cells with both transforming and nontransforming isolates with and without the addition of 5% complement.

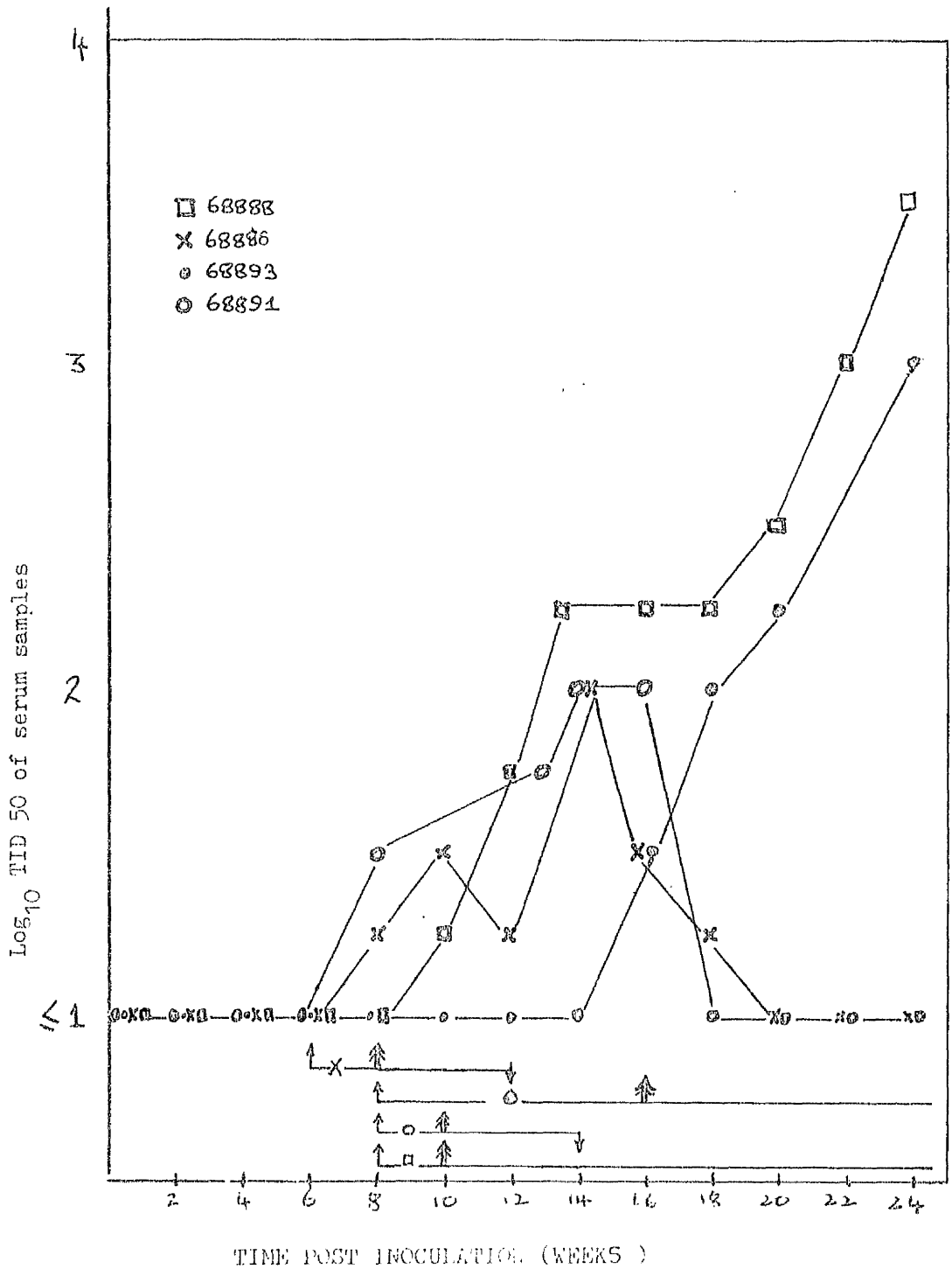


Figure 28: Transformation Inhibition titres of experimental calf sera in relation to post inoculation time development and regression of fibropapillomas

first appearance of fibropapillomas

first histological evidence of cell mediated immune regression

(if applicable) rejection of fibropapillomas complete

8.3 Results

Figure 28 illustrates the TID 50 titres of sera from cattle experimentally infected with various BPV isolates. Full details of the transmission data of these calves appears in Chapter 3 of this thesis. Calves which developed multiple fibropapillomas all showed detectable transformation inhibitory (TI) activity in their sera. This TI activity was first detected in sera at the time of first appearance of masses of lymphocytes in the fibroma component indicating the onset of cell mediated regression of lesions. Calves 68891 and 68886 both successfully rejected their fibropapillomas and TI titres fell to undetectable levels 4-8 weeks following complete rejection. Calves 68893 and 68888 mounted a rejection response but failed to reject fibropapillomas successfully and TI titres continued to show a progressive increase until post-mortem at 24 weeks. Serum samples from calves inoculated with transformed cells (45, 81, 83, TFBSC(i), TFBMC), control cells (FBSC, FBMC) and teat rice grain virus (68882, 84) failed to show detectable TI titres. Calves 82 and 68882 both showed small fibropapilloma lesions which were removed for biopsy early in their development. For a more complete description of the transmission experiments refer to Chapters 3 and 5. Calves 68891 and 82 both showed meningiomas at post-mortem 35 weeks post inoculation. Histological examination failed to show evidence of cell mediated rejection. TI assay of serum samples taken at PM failed to show detectable TI activity. All titres shown on figure 28 are the means of four replicant assays.

Figure 29 shows the effect of varying virus and complement concentrations on the TID 50 of one serum sample diluted precisely to the previously determined TID 50 using 5% complement and 100 transforming units of virus per well. Each point on the graph represents the result of twelve replicate titrations. At very high concentrations of BPV the TI effect of the serum is reduced, however, over a wide range (2.5 log units) of concentrations, virus dilution does not alter the TI of the serum. When insufficient virus is present to transform the

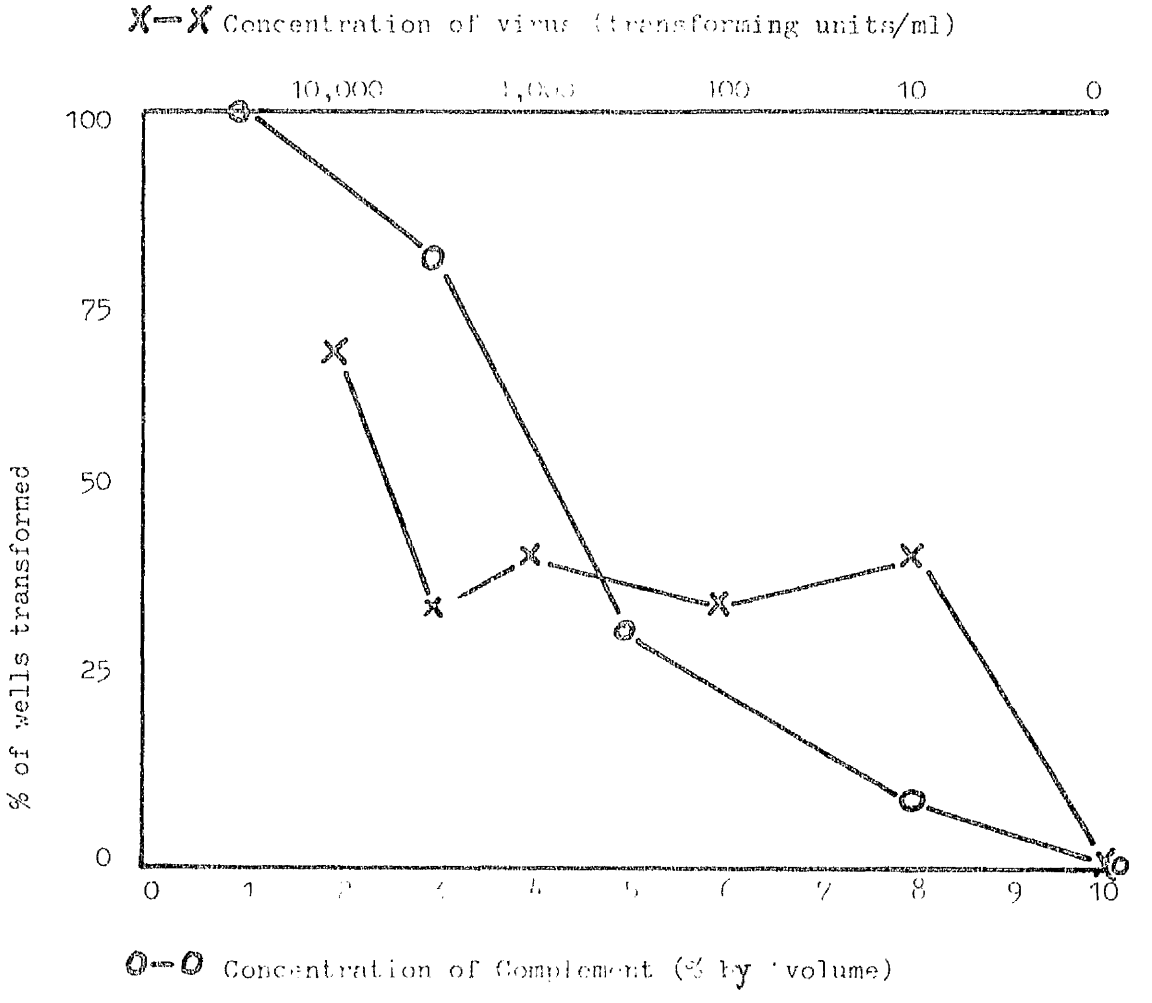


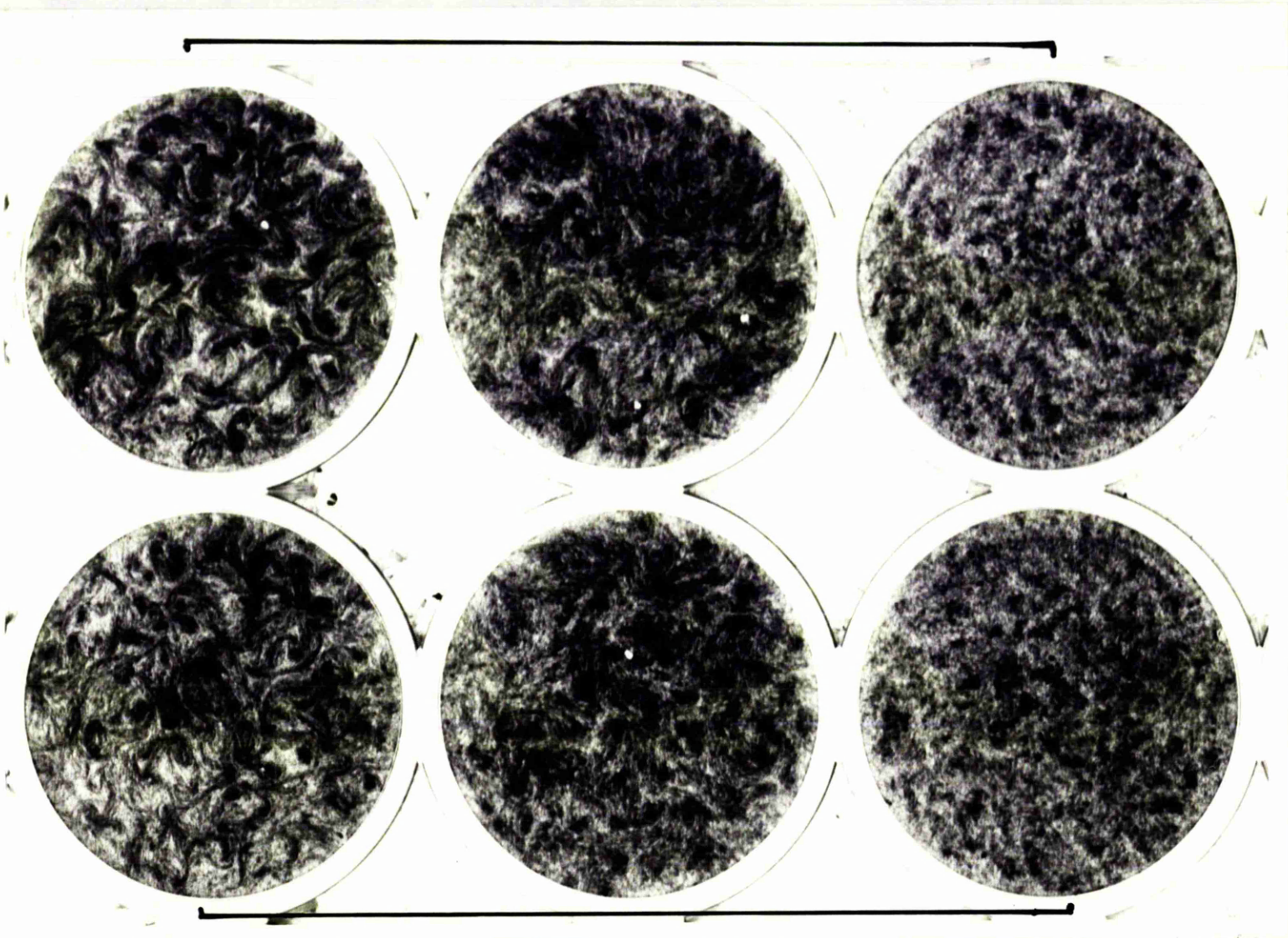
Figure 29: The effect of varying concentrations of virus and complement on transformation inhibition using one high titre serum (0889 week 24)

cells then obviously the serum cannot exert an effect. In contrast, varying complement concentrations from 1 to 10% resulted in marked variations in serum TI effect. At 1% there was no TI while at 10% TI was complete

Figure 31 illustrates the morphological changes when a non-transforming fibropapilloma virus isolate is inoculated into foetal bovine cultures. This effect was identical when skin, conjunctiva, palate and meningeal cultures were used. When high concentrations of a transforming virus isolate are used in conjunction with high titre antiserum, identical morphological changes are seen. The morphological changes can be reproduced only with high virus concentrations with antibody. The changes appear between days 5-7 post inoculation and are either masked or disappear by days 8-10.

Table 32 shows the effect of prior adsorbtion of one high titre serum (68888 W24) using various BPV-related suspensions. Significantly reduced titres ($P < 0.01$) resulted following adsorbtion with $CsCl$ purified fibropapilloma virus and tissue pieces or cells of cutaneous fibropapilloma; fibroma; BPV induced meningioma; and fibropapilloma derived BPV-transformed foetal bovine conjunctiva cells. Slightly reduced titres (not significant) were observed following adsorbtion with normal foetal tissue; anogenital fibropapilloma 10% w/v clarified suspension; and teat papilloma tissue pieces. All other adsorbants did not reduce the TI titre of the serum.

Figure 30 illustrates a typical transformation assay plate for comparison with Figure 27. Repeated attempts to detect interference between non-transforming and transforming virus isolates failed. Repeated attempts to inhibit the phenotypic appearance of transformed cultures using high titre TI serum were unsuccessful even though adsorbtion with transformed cells did significantly reduce antibody titres. At very high concentrations of antiserum (eg neat and 1:10 dilutions) there was a marked inhibition of cell growth with cells becoming enlarged, vacuolated with an almost cuboidal appearance. However, in those wells where cells continued to grow, they retained their transformed morphology.



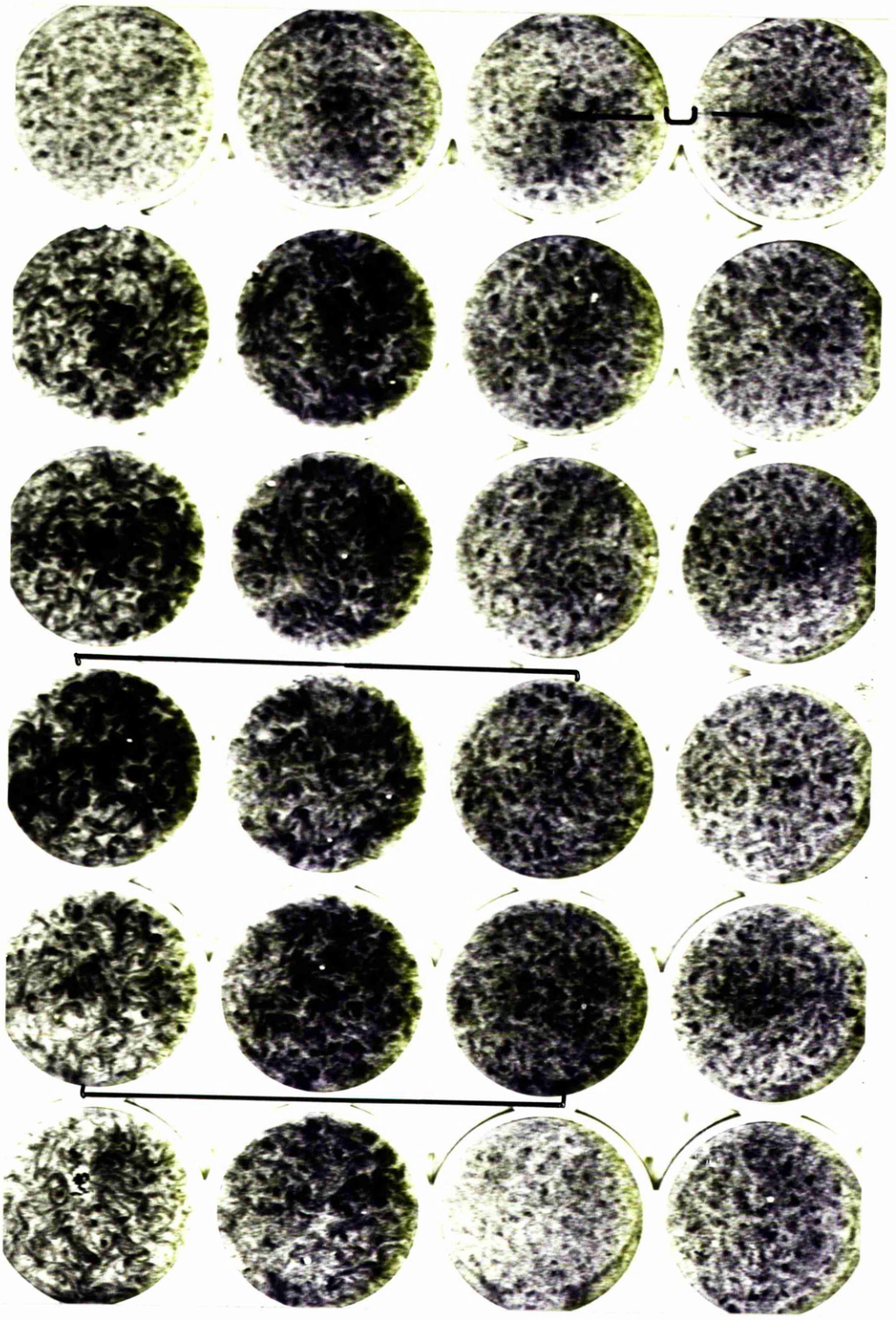


Figure 30 Transformation assay plate for bovine papilloma virus. a) photograph of whole plate - row C controls; darker wells show transformation wisps.

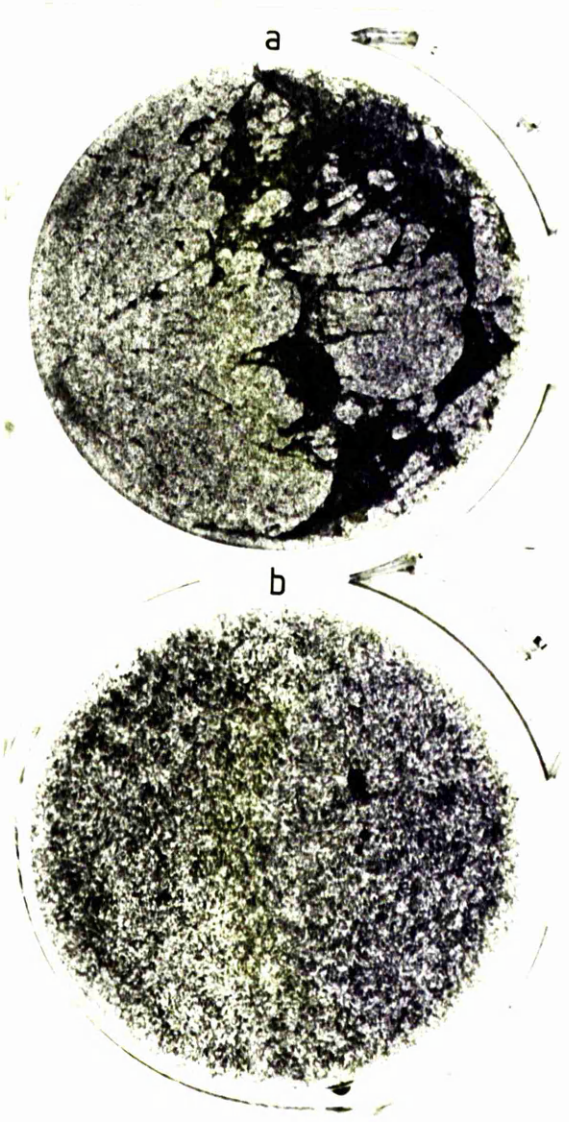


Figure 31 Morphological changes seen in vitro when high concentrations of virus and high titre TI serum are used. a) affected b) control wells. These changes are identical to those produced by non-transforming fibropapilloma isolates.

8.4 Discussion

Antivirion (v) antibodies have been reported for bovine papilloma virus and these have been used to visualise virus within the nuclei of superficial epithelial cells in fibropapillomas (Smithies and Olson, 1964). The same authors report that V-antigen has never been detected in the fibroma cells and neither has virus been seen in these cells under the EM. Lee and Olson (1969) reported a gel diffusion precipitin test for bovine papilloma virus. Using identical methods, as well as those reported by Koller et al., (1974) and Almeida and Goffe (1965), no precipitin lines developed when the sera used in this chapter were tested. Agar and agarose concentrations were varied from 0.5 to 2%, buffers of phosphate and citrate were used. Antigen preparations included homogenised fibropapilloma tissue, clarified 20% and 10% w/v suspensions and CsCl purified and concentrated BPV. Antigen preparations were variously treated with hypotonic saline, sodium dodec yl sulphate, 30 second, 2 and 10 minute ultrasonication without avail. The conclusion is that no anti-V antibodies, detectable by immunodiffusion were present in the calf sera tested.

Lee and Olson (1969b) reported that, in experimental calves, anti-V antibodies first appeared between 1 and 2 weeks following inoculation and persisted for up to 16 weeks. Anti-V antibody presence in sera could not be correlated with the growth or regression of fibropapillomas.

Barthold and Olson (1974b) detected a membrane antigen to BPV-induced fibroma cells using indirect immuno-fluorescence on live unfixed fibroma cells in vitro. They reported that adsorbtion with tumour cells removed the antibody activity in test sera. The cultured fibroma cells were negative for V-antigen and EM examination failed to detect BPV particles. Sequential antibody titres correlated with fibropapilloma onset but not with progression or regression of tumour growth. Maximum antibody titre obtained using the indirect immunofluorescence test was 32. Of seven calves sampled, 3 developed persistent fibropapillomas, 3 developed

small tumours which rapidly regressed and 1 showed no response to inoculation. In two calves where fibroma development was minimal and regression was complete 8 weeks post inoculation, antibody persisted for a further 9 months at peak titre values. Barthold and Olson (1974b) did not attempt to correlate the histological onset of regression (typified by lymphocyte accumulation - Chapter 3) with antibody titres.

For calves 68888, 68891 and 68886 it would appear superficially that antibody response correlates with the first appearance of fibropapillomas; however, closer inspection of these calves together with calf 68893 (Figure 28) shows a positive correlation between first appearance of lymphocyte accumulation in tumours and detectable TI antibodies in the serum. Only calf 68891 showed a low titre with the appearance of lymphocytes in the tumour biopsy two weeks later. This may have been a sampling error.

The results reported here indicate that TI antibody titres persist only 4-8 weeks following complete regression. These findings conflict with those of Barthold and Olson (1974b) but are in general agreement with those for TSTA in SV40 induced tumours (Chang et al., 1977) and the findings with human papilloma related cell mediated immunity reported by Lee and Eisinger (1977). The half-life of immunoglobulins in more than 100 day old calves has been reported to be 22.8 days (Cunningham, 1978) and this estimate is in accordance with the findings in this chapter. Black et al., (1963) have reported transformation inhibition titres as high as 5.12×10^3 but make no comment about the complement dependence of their test system; however they used non-heat inactivated foetal bovine serum (FBS) in their growth medium. Experience has shown that this source, if fresh can alone supply enough complement for transformation inhibition. Since complement levels in FBS are highly variable, the work reported here has been conducted using a standard source of complement to ensure comparability of titres obtained in sequential assays.

Adsorbtion experiments reported here are in agreement with those of Barthold and Olson (1974b). They provide further evidence that while TI antibody is not directed against purified or clarified BPV preparations from a variety of sources (Table 32), it is directed against in vivo and in vitro cells transformed by fibropapilloma virus. The absence of a similar response to tissue suspensions of teat rice grain lesions and teat papillomas is in accord with the non-cross reactivity between the TSTA's of JCV, BKV and SV40 induced tumour cells (Padgett et al., 1977; Padgett and Walker, 1976). Related but different papova viruses appear to have distinct TSTA's. The reduction of TI titre by adsorbtion with purified but not clarified cutaneous fibropapilloma virus may simply be a reflection of the vastly greater virus concentration in the former than the latter. On a gram equivalent weight of tumour tissue, the purified extract contained 10^3 times greater virus than the clarified suspension. This would over-ride any equivalence between antigen and antibody as indicated in Figure 29.

Although meningoma tissue suspensions reduced TI titre in vitro, this observation does not agree with the in vivo data. At the time of post mortem (35 weeks) both calves 82 and 68891 showed large meningiomas but sera taken at the same time contained no detectible TI activity. Histological examination of tumours showed no evidence of a rejection response, even though both calves had developed and rejected cutaneous fibropapillomas five months previously. Whether this is due to an effective blood-brain barrier preventing tumour cell escape to the peripheral circulation must await further investigation.

The absence of TI activity in sera of calves inoculated with transformed cells may be due to the relative insensitivity of the test system since these calves were shown to be more resistant to challenge than control cell inoculated calves in vivo (Chapter 3).

The presence of TI antiserum and complement does not cause previously transformed cells to revert to normal growth morphology. At high concentrations, there is an apparent

cytotoxic effect, and adsorbtion with transformed cells does reduce TI titre. The absence of a reversion to normal morphology may be due to antigen excess in less than cytotoxic antibody-complement concentrations.

8.5 SUMMARY

This chapter has presented evidence for a complement-dependent transformation inhibition assay which detects antibodies directed against BPV-transformed cells in vitro and BPV induced tumours in vivo. First appearance of antibody occurs following fibropapilloma onset and is correlated with the histological appearance of lymphocyte accumulation within tumours. Following complete tumour regression, TI antibodies remain detectable for 4-8 weeks. No TI antibodies could be detected in calves infected with teat rice grain lesion BPV, transformed cells or control cells. TI antibody titre could be reduced by adsorbtion with BPV-induced meningioma tissue in vitro but TI antibodies were not correlated with the presence of meningiomas in vivo.

Adsorbtion of high titre TI antisera with teat rice grain lesion tissue and teat papilloma tissue did not reduce TI activity, while adsorbtion with BPV induced fibropapilloma, fibroma and meningioma tissue and BPV transformed cells caused significant titre reductions. The TI assay is more sensitive than but appears to be detecting similar anti-cell antibodies to the indirect immunofluorescence assay reported by Barthold and Olson (1974b).

CHAPTER 9 ATTEMPTS TO GROW BOVINE PAPILLOMA VIRUS IN VITRO

9.1 Introduction

Many reports appear in the literature claiming to have grown papilloma viruses in vitro (Mendelson and Kligman, 1961; Macpherson, 1962; Morgan and Balduzzi, 1964; Oro zlan and Rich, 1964; Noyes, 1965; Eisinger et al., 1975; and Hadden, 1975). Olson et al. (1960) claimed to have grown BPV in the chorioallantoic membrane of fertile chicken eggs (see also Bagdonas and Olson, 1954). All of these reports await confirmation. In Chapter 1.4.8, this author concluded that HPV and BPV have indeed been cultivated in vitro, but inefficiently and under exceptional circumstances. There is still, however, no reliable method of cultivating several of the polyoma viruses as well as any papillomavirus in vitro.

This chapter reports on attempts made to grow BPV in vitro on all primary foetal bovine cell cultures established in the laboratory.

9.2 Materials and Methods

9.2.1 Cell Cultures

Since all papilloma viruses appear intranuclearly only in the nutrient starved upper layers of the epithelium, a standard method was developed to test cultures for in vitro BPV replication. Since transformation-sensitivity is not always related to permissivity for replication, all 44 cultures established and reported in Chapter 7 were tested using this method.

Each culture was suspended using trypsin/versine and 5×10^6 cells plus the equivalent of 10^3 transforming units of fibropapilloma derived BPV were inoculated in 100 ml. BME with 10% added FBS. Burlers were rotated 3-4 times per hour in a hot room held at $37^{\circ}C$. At weekly intervals, the medium was replaced with 100 ml. fresh BME with 10% FBS. for a minimum of 8 weeks. Cells were not subdivided or otherwise treated during the eight weeks.

9.2.2 Electron Microscopy

Collected spent media were precipitated with equal volume added saturated ammonium sulphate at $4^{\circ}C$. The precipitate was suspended in 10 ml PBS before being subjected to differential centrifugation and C_sC1 purification as described earlier for virus preparation (Chapter 7). CsC1 fractions around 1.36 were collected, diluted to ten ml with PBS and then centrifuged for 90 minutes at 37,000 r.p.m. on an SW41 rotor in a Beckman

ultracentrifuge. Pellets were resuspended in 0.1 ml PBS prior to negative staining for EM detection of virus particles. In all, 352 spent medium samples were tested in this fashion.

Four transformation sensitive cultures were prepared as described under 9.2.1 but with paired control burlers, and twenty 5 ml plastic bottles paired with control and transformed culture containing burlers. Cells were removed from these bottles using glass beads at selected times post inoculation and further processed for thin section EM examination. The time intervals selected were 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32 hours; 2, 4, 6, 8, 10, 13, 17, 20, 23, 24, 28 days post inoculation. One of these cultures (5M) showed presence of virus in the medium 28 days post inoculation, and all thin section cell samples of this culture were examined in detail under the EM.

One culture (11S) was examined identically to that outlined earlier. Two burlers were set up as described in 9.2.1 with the exception that to each medium was added 0.5 millicuries of tritiated thymidine. Scintillation counts were made of all CsCl fractions of the eight gradients containing spent medium extracts. Gradient samples around refractive index 1.36 were examined under the EM for BPV detection. In this experiment cells and debris collected in the spent medium were subjected to three freeze-thaw cycles and examined under the EM (see section 2.2.7). Prior experimental treatment of the same cells had shown a concentration of 2 mg/ml Bleomycin to be cytostatic but not cytotoxic. 2 mg/ml of Bleomycin was added to the medium of one burler 21 days post inoculation.

9.2.3 Transformation Assay

In the three cases where BPV was found in the medium, the samples were assayed as described in Chapter 7 to determine the Log TD50.

9.2.4 Other Investigations

The spent medium from all transformed and control cultures maintained in the laboratory were collected and stored according to culture of origin. Medium from over 2,000 subdivisions (comprising 40 litres) conducted routinely was examined in this way. Pooled spent medium was treated as described in 9.2.2 before being examined for the presence of BPV under negative staining in the EM.

9.3 Results

EM examination of all spent medium from routinely subdivided transformed and control cultures failed to demonstrate BPV particles.

One culture, 2M, of thirty nine tested specifically for BPV growth showed the presence of BPV particles under the EM in medium collected 24 days post inoculation. Figure 32 is an electronmicrograph of this isolate. Of interest is the fact that this culture, unlike the others, was neglected. Since it was established out of synchrony with other cultures, it received it's first medium at 3 days, the second at 10 days and the third at 14 days. It was this latter medium collected 24 days post inoculation which was found to contain the BPV illustrated in Figure 32. The culture had spent 10 days without medium in contrast to the normal seven for the others.

One culture (5M) of four sampled serially and tested for BPV growth showed the presence of BPV in the medium collected 28 days post inoculation. This culture was found to be contaminated with Staphlococcal bacteria at the same time. Detailed EM examination of serially sampled thin sections showed BPV presence around the cells; intra-cytoplasmic BPV, both enclosed in vesicles and unenclosed; and between the two laminae of the nuclear envelope. Virus presence could only be detected up till 4 days post inoculation. In all of the subsequent samples, no BPV could be detected on thin section examination. No intranuclear virus particles were seen although a small proportion of the cells showed gross degenerative changes with associated nuclear abnormalities.

In one culture (11S), BPV particles were seen in negative stain preparations of CsCl purified spent medium collected 28 days post inoculation from the Bleomycin treated burler but not from the untreated burler. Freeze-thaw samples of cells and cell debris also showed the presence of BPV particles under the EM. Figure 33 shows the radioactivity expressed in counts per minute of CsCl gradients of both treated and untreated spent medium samples.

Transformation assay of all three BPV samples found in this work showed a Log_{10} TD50 of 4.1 in the 2M production, compared with 4.3 of the original inoculation; a titre of 2.7 in the 5M production, compared with a titre of 4.2 in the original inoculum.

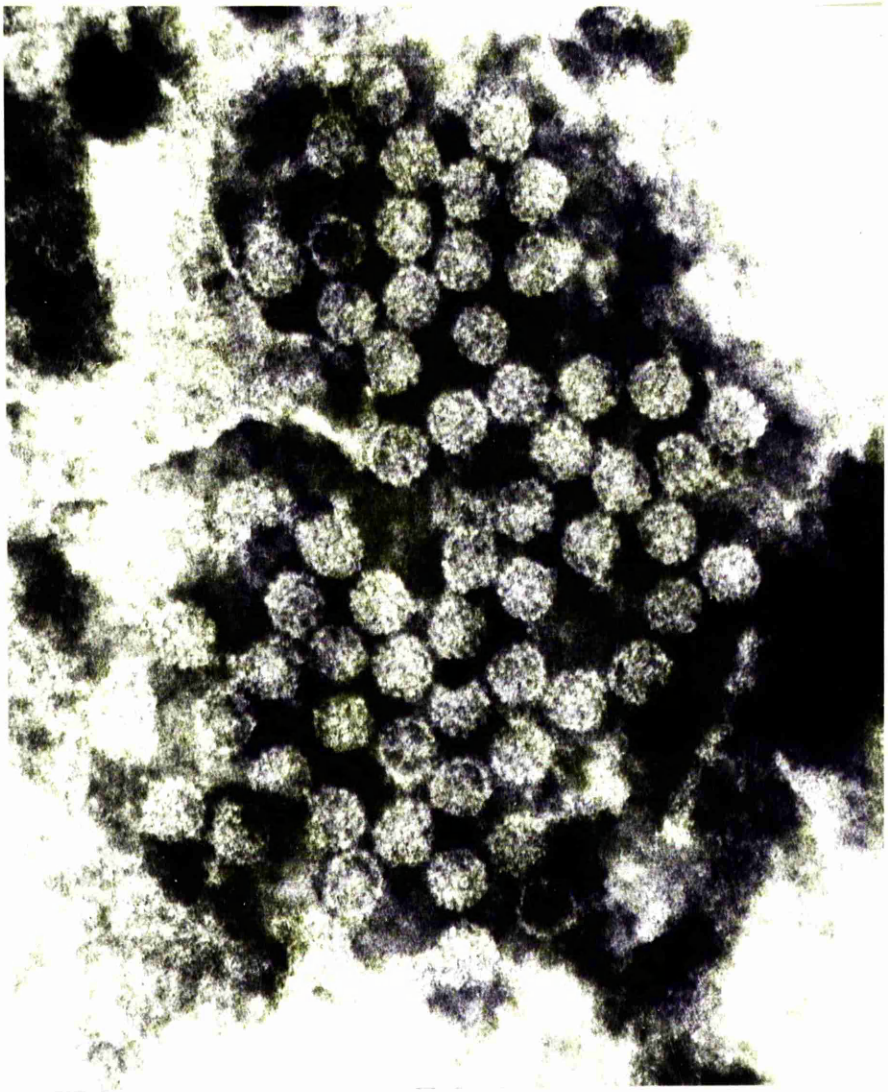


Figure 32 Electronmicrograph of BPV present in culture medium 24 days post inoculation magnification approximately x 180,000.

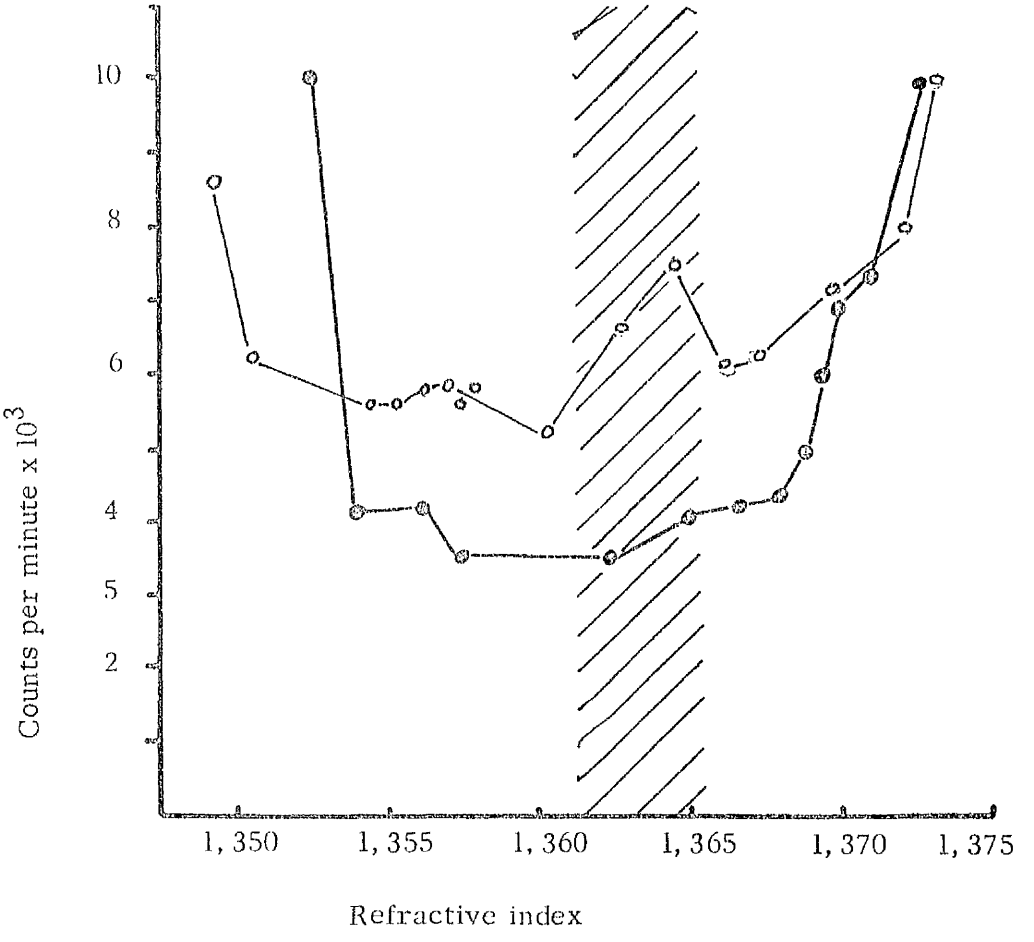


Figure 33 Radioactivity in CS Cl gradients of medium samples from Bleomycin-treated and untreated BPV infected cell cultures

treated

untreated

E. M. determined BPV positive area in treated gradient

note: each point represents the mean of four readings.

9.4 Discussion

Although many reports in the literature claim to have grown human papilloma virus or bovine papilloma virus (see introduction) there exists no reliable technique for the in vitro growth of any papilloma virus and several polyomavi ruses. The evidence in the literature is sufficiently convincing to conclude that human papilloma virus has been cultivated in vitro - but very rarely and only under specialised conditions. (for example Eisinger et al., 1975)

The results of this chapter tell a similar story. Under normal cultural conditions, no culture showed BPV production. However under conditions where cells remained in situ for up to two months, three of 44 cultures tested showed the presence of BPV in medium collected at 24 or 28 days post-inoculation. That the virus present in the medium was BPV is supported by the EM size and morphology; the CsCl density; and the in vitro transformation by two of the three isolates. Support for the fact that the virus was produced by the cells comes from the radioactivity peak at the BPV refractive index of 1.36 indicating that the viral DNA had incorporated tritiated thymidine which can only occur during viral production. The inability to detect BPV in thin section samples may be a reflection of the very small number of cells which were actively permissive for DNA replication. Comparative transformation titres of inoculated virus and produced virus indicate that the virus produced was in very low quantities. Further evidence that the virus was actually produced in vitro comes from the dilution factors involved. By the time virus was found in spent medium, the original inoculum had been diluted by successive medium changes by a factor of 10^8. It was considered possible that the virus could be absorbed but not degraded by the cells and then released on their death. The radiolabelling experiment mitigates against this, however.

The work involved in carrying out the experiments was considerable. The results are at best a small contribution to possible avenues for further work. They are by no means conclusive, and will not be published except in this thesis. The two factors which all three productive "bursts" have in common are (1) prolonged contact inhibition and (2) severe stress either through lack of nutrients, bacterial contamination or Bleomycin treatment.

9.5 Summary

Three of forty-four cultures tested showed evidence of in vitro viral replication between 24 - 28 days following BPV infection. These cultures were subjected to prolonged contact inhibition and severe stress prior to the appearance of BPV in the medium. Detailed thin section EM examination failed to detect viral particles in the cells from one culture showing BPV in the medium and freeze-thaw samples of cellular debris. Despite the amount of work involved, the results are inconclusive and may only serve as suggestive avenues for further research effort.

CHAPTER 10 GENERAL DISCUSSION AND MAIN THESIS FINDINGS

No previous surveys of teat or cutaneous BPV-related lesions have been published. A survey of alimentary papillomatosis has reported an overall prevalence of 19% (Jarrett et al., 1978). In the human, prevalence figures for papillomatosis, focal epithelial hyperplasia and other HPV-related lesions vary widely according to sex, age and geographical parameters (Section 1.3). The teat survey reported in Chapter 2 provides an overall prevalence of 36% in 721 cattle inspected. There were significant sex differences in the prevalence of the disease and nulliparous female cattle had significantly fewer teats affected than parous female cattle. These findings contrast with those of Jarrett et al. (1978) who reported no significant sex differences in the prevalence of alimentary lesions. The high prevalence of teat lesions was surprising because concurrent examination of the rest of the integument showed a cutaneous fibropapilloma prevalence of 2.7% . These differences may have been due to animal factors (eg. the teat may be an immunologically deprived site, or, the teat is more regularly traumatised than other parts of the integument) or viral differences. Histological examination of individual lesions showed that teat lesions could be divided into three broad categories - fronded true papilloma, focal epithelial hyperplastic lesions (rice grain) and fibropapilloma (Chapter 3). The epidemiological data presented in Chapter 2 indicat that at least two virus/host interactions occurred. The teat rice grain lesions were significantly different in distribution both in shape of curve and number of lesions. Differences were also detected in virus particle diameter and apparent molecular weight of major viral capsid protein.

When single case, simple lesion type BPV extracts were transmitted to experimental calves, morphological and developmental differences between the transmitted lesions were correlated with the lesion type of the original extracts. BPV was extracted from all transmitted lesion types. Koch's postulates were thus fulfilled- and four separable BPV extracts were identified, - cutaneous, anogenital fibropapilloma; true papilloma and rice grain lesion. Challenge experiments revealed that in vivo resistance to reinfection with fibropapilloma extracts was not accompanied by resistance to infection with BPV from papilloma or rice grain lesions. Similarly regression of fibropapilloma on calves was not accompanied by regression of other lesion types.

In Chapter 5, evidence is provided for differences in ability to induce meningiomas of BPV extracts. Although all virus intracerebrally inoculated calves showed lesions at intradermal inoculation sites, only

those calves inoculated with fibropapilloma derived BPV developed meningiomas. The teat rice-grain lesion derived BPV inoculated calf did not show meningioma development.

Even though the total number of experimental calves used was small, the above mentioned experiments suggest two broad virus categories exist. One type with a cellular trophism which includes both epithelial and mesenchymal tissues and the other which is limited to epithelial tissues only. Within these two categories, other differences exist both in the type of epithelial tumour produced and the degree of mesenchymal fibroma involvement.

Although the literature now contains ample evidence for the existence of separate human papilloma viruses, some associated with specific disease syndromes - only one paper reports the existence of a non fibropapilloma lesion in cattle (Barthold et al., 1974).

The terms used in this thesis to denote different teat lesions are those widely used, but not reported in the literature. It is probable that the terms were first used by Dr. Ian Lauder (M. Murray - personal communication). Therefore, the existence of different teat lesion types in cattle has been widely recognised, even if not duly reported, in this department. Differential experimental oncogenicity among isolates of the same papovavirus has only recently been shown (Padgett et al., 1977) for JCV virus, however the existence of different oral cutaneous papilloma viruses in the same species has been generally accepted for a long time (eg. human, simian, equine, canine and rabbit species). The bovine species can now be placed with these others in that it possesses different papillomaviruses.

Further evidence for the existence of different papillomaviruses comes from the invitro work reported in Chapters 7 and 8. Fibropapilloma derived BPV from cutaneous, anogenital, alimentary and teat lesions transform foetal bovine cells in vitro, while BPV from papilloma and teat rice grain lesions do not. Inhibition of in vitro transformation is complement dependant. Transformation inhibitory (TI) activity could be detected in sera from cattle with fibropapillomas but not in cattle with rice grain lesions.

In vitro transformed cells were tumorigenic in nude mice but not cattle. However they did increase in vivo bovine resistance to challenge with fibropapilloma derived BPV but not papilloma or rice grain lesion derived BPV.

This thesis thus provides histological, epidemological, immunological (in vivo and in vitro),physical and biochemical evidence for the

existence of different types of BPV. Further work on BPV-DNA will be able to define these differences at the molecular level. While completely speculative, it is possible that BPV differences reported here could have relevance to the question of which virus type (s) are involved in the malignant BPV-related lesions seen in the bovine upper alimentary tract.

In vitro transformation by BPV has been reported earlier (section 1.4.7) and this thesis extends knowledge in defining the precision and sensitivity of this phenomenon for use as an alternative method of quantitating fibropapilloma BPV biologically. The transformation-sensitive tissues of the bovine foetus are also reported as meninges, conjunctiva, palate and skin. Transformation titres varied with virus concentrations and not foetus age or tissue type from which the culture was isolated. Transformed cells showed heritable changes, an increased lifespan and were tumorigenic in nude mice. They also increased in vivo resistance of calves to subsequent challenge with fibropapilloma BPV.

Transformation inhibition (TI) has been shown to be a complement-dependant anti-transformed cell phenomenon in this thesis. Although reported earlier (Black et al., 1963), this thesis extends knowledge by defining the precise requirements for the successful use of TI as an assay system measuring anti BPV-transformed cell antibodies in the sera of calves showing certain BPV-related tumours. The cellular antigens detected using the TI assay appear to be similar to those reported by Barthold and Olson (1974 b) and may be TSTA's as described in other papovavirus systems (section 1.4.1). TI antibodies were first detected in the sera of fibropapilloma bearing calves coincident with the first appearance of lymphocyte accumulation in tissue biopsy samples being detected. TI antibodies persisted for 4-8 weeks following complete regression of fibropapillomas.

The detection of BPV particles in extracts of milk purchased from retail outlets prompted an investigation of time-temperature treatment effect on BPV in vivo and in vitro. Pasteurisation was shown in vivo and in vitro to have no effect on BPV activity. In vitro, complete inactivation of BPV transformation occurred after 30 minutes at 80^{o}C and a tenfold reduction in titre followed treatment for 4 hours at 60^{o}C. BPV is thus similar to most, but not all papovaviruses as listed in 1.6.3 in its relative heat stability. The results of this work, coupled with the histological and gross similarity of lesions of the bovine teat and certain oral lesions in man make further investigation of possible

human lesions related to BPV of distinct importance even though literature evidence for BPV inf ection in man is equivocal (section 6.4).

Substantial effort made to find a method for culturing BPV in vitro was largely unrewarded. This author can report three occasions of 44 attempts where BPV was found in culture medium between 24 - 28 days post inoculation. It appears that prolonged contact-inhibition coupled with severe stress immediately prior to virus release were contributory factors. As with other authors (section 9.4), this work is far from conclusive and, at best, may provide ideas for further research effort.

The main findings of the work reported in this thesis are as follows:

1) The development and characterisation of an in vitro transformation assay for fibropapilloma derived BPV.
2) The development of a complement dependant transformation-inhibition assay detecting anti-fibropapilloma derived BPV-transformed cell antibodies.
3) The isolation and transmission of different bovine papilloma viruses. These viruses are different in physical, biochemical, immunological and biological characteristics.
4) The definition of the heat-sensitivity of BPV in vitro and in vivo.
5) The abattoir survey of teat lesions with the discovery of high prevalences and particular epidemiological features of different lesion types.
6) The finding that fibropapilloma derived BPV-transformed cells are tumourigenic in nude mice but not cattle. However, cattle are more resistant to subsequent challenge with fibropapilloma derived BPV following inoculation of transformed cells.
7) The discovery of certain factors which may be involved in a future search for a method of in vitro cultivation of BPV.

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APPENDIX A - RESULTS OF ABBATTOIR PAPILLOMA SURVEY

(i) WHOLE ANIMAL DATA - SUMMARY

SUBSURVEY	NUMBER CATTLE EXAMINED	CATTLE AFFECTED	%	TEATS AFFECTED	%
1-9-77	63 Heifer	21	33.3	41	65.1
	49 Cow	22	44.9	46	93.9
	39 Male	10	25.6	14	35.9
8-9-77	43 Heifer	14	32.6	31	72.1
	102 Cow	45	44.1	117	115.0
	28 Male	6	21.4	7	25.0
15-9-77	27 Heifer	11	40.7	28	104.0
	68 Cow	34	50.0	77	113.0
	38 Male	6	15.8	10	26.0
29-9-77	37 Heifer	10	27.0	20	54.0
	41 Cow	20	48.8	49	119.0
	27 Male	4	14.8	4	14.8
6-10-77	56 Heifer	12	21.4	21	37.5
	68 Cow	36	52.9	76	111.8
	35 Male	9	25.7	14	40.0

Note: only 3 animals had scrotal papillomas; rest were on teats of animals of both sexes. Scrotal papillomas not included in summary table.

APPENDIX A

(ii) Whole animal data - detail

* 1-9-77		1-9-77		1-9-77		1-9-77		1-9-77		1-9-77	
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
1H	0	17C	1	33H	0	49H	1	65C	0	81S	0
2H	0	18C	0	34H	0	50H	0	66C	0	82S	0
3H	0	19H	0	35H	0	51C	2	67H	0	83S	0
4H	0	20H	0	36H	1	52C	0	68H	0	84S	0
5H	0	21H	0	37H	0	53C	0	69H	0	85S	0
6H	0	22H	0	38H	0	54C	4	70H	0	86S	3
7H	0	23H	0	39H	0	55C	1	71H	0	87S	0
8H	0	24C	3	40H	0	56C	3	72C	0	88S	1
9H	0	25C	2	41S	Scrotum	57H	0	73C	2	89S	0
10H	0	26C	0	42S	0	58H	0	74C	0	90S	0
11H	2	27C	0	43S	0	59H	2	75C	0	91S	0
12H	0	28C	1	44S	Scrotum	60H	0	76C	0	92S	2
13H	0	29C	0	45S	0	61H	0	77C	0	93S	0
14C	1	30C	0	46S	0	62H	2	78S	0	94C	0
15C	0	31S	0	47H	1	63C	0	79S	0	95C	0
16C	0	32H	1	48H	0	64C	2	80S	1	96C	0

APPENDIX A

(ii) Whole animal data - detail

* 1-9-77		1-9-77		1-9-77		1-9-77		8-9-77		8-9-77	
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
97C	0	113S	0	128S	1	143C	3	1C	2	17C	0
98C	0	114S	0	129S	0	144C	2	2C	0	18C	0
99C	3	115S	0	130S	0	145C	0	3S	0	19C	0
100C	0	116H	0	131S	0	146C	0	4S	0	20C	1
101C	1	117H	4	132S	0	147C	2	5S	1	21C	0
102C	2	118H	2	133H	4	148C	1	6S	0	22C	0
103C	2	119H	1	134H	1	149H	2	7S	0	23C	0
104S	1	120H	1	135H	2	150H	3	8S	1	24C	3
105S	1	121C	2	136H	3	151H	0	9S	0	25C	0
106S	0	122C	0	137H	1			10S	0	26C	2
107S	2	123S	0	138H	2			11S	0	27C	0
108H	1	124S	0	139H	0			12S	0	28C	0
109H	0	125S	0	140C	0			13S	0	29C	2
110H	0	126S	0	141C	4			14S	0	30C	1
111H	4	127S	0	142C	2			15S	0	31C	0
112S	2							16C	2	32C	0

* subsurvey date; S = steer; C = cow; H = heifer; B = bull

APPENDIX A

(ii) Whole animal data - detail

* 8-9-77		8-9-77		8-9-77		8-9-77		8-9-77		8-9-77	
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
33H	0	49S	0	65H	4	81C	0	97C	4	113C	1
34H	2	50S	0	66H	4	82C	2	98C	4	114C	0
35H	1	51H	1	67H	0	83C	0	99H	0	115C	0
36H	0	52H	0	68C	0	84C	0	100H	2	116C	0
37H	0	53H	0	69C	4	85H	0	101H	0	117C	3
38H	0	54H	1	70C	0	86H	0	102H	0	118C	0
39H	1	55C	4	71C	1	87H	0	103C	0	119C	2
40H	0	56C	0	72C	0	88H	0	104C	3	120C	0
41C	0	57S	1	73C	0	89H	2	105C	2	121C	2
42C	2	58S	0	74H	3	90H	0	106C	0	122C	1
43C	1	59C	0	75H	0	91B	Scrotum	107C	0	123H	0
44C	0	60C	4	76H	0	92C	0	108C	0	124H	0
45C	0	61C	4	77H	0	93C	4	109C	0	125H	0
46B	0	62C	0	78H	0	94H	0	110C	0	126H	0
47B	0	63C	0	79C	4	95H	0	111H	0	127H	0
48B	0	64H	0	80C	0	96C	0	112C	1	128C	0

* subsurvey date ; S = steer;, C = cow; H = heifer ; B = bull

APPENDIX A

(ii) Whole animal data - detail

* 8-9-77		8-9-77		8-9-77		15-9-77		15-9-77		15-9-77	
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
129C	2	146H	4	163C	4	1H	0	17H	4	34C	0
130C	0	147H	1	164C	0	2H	1	18H	1	35C	2
131C	0	148C	1	165C	0	3H	0	19C	0	36C	1
132C	0	149C	4	166C	0	4C	1	20H	4	37C	0
133C	3	150C	2	167C	4	5C	0	21H	3	38C	0
134C	0	151C	0	168C	0	6C	1	22H	2	39C	0
135H	2	152C	4	169C	0	7C	0	23S	0	40C	2
136H	0	153C	3	170C	1	8C	2	24C	4	41C	0
137H	0	154C	0	171C	0	9C	1	25C	3	42C	0
138S	0	155C	2	172C	2	10C	0	26C	0	43C	0
139S	1	156C	2	173C	0	11S	0	27C	2	44C	0
140S	0	157C	3	174C	0	12S	0	28C	0	45C	1
141S	0	158C	2			13S	0	29C	4	46C	0
142S	0	159C	4			14S	0	30H	4	47H	0
143S	0	160C	4			15S	0	31H	0	48H	0
144B	2	161C	4			16C	0	32H	0	49S	0
145H	3	162C	0					33C	4	50S	0

APPENDIX A

(ii) Whole animal data - detail

* 15-9-77		15-9-77		15-9-77		15-9-77		15-9-77		29-9-77	
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
51S	0	67C	0	83H	1	99C	1	115H	3	1C	1
52S	1	68C	3	84H	0	100C	2	116H	0	2C	0
53S	0	69S	0	85H	0	101C	3	117C	2	3C	0
54H	0	70S	0	86S	0	102C	3	118C	0	4C	4
55S	1	71S	0	87S	0	103C	1	119C	0	5C	0
56S	0	72C	0	88S	0	104C	0	120C	0	6C	1
57S	0	73C	0	89H	2	105C	0	121C	1	7C	0
58S	0	74C	0	90H	0	106C	0	122C	0	8C	3
59S	2	75C	4	91H	0	107C	2	123C	0	9C	0
60S	0	76C	4	92S	0	108C	0			10C	0
61S	0	77C	0	93S	0	109C	4			11C	0
62S	0	78C	0	94S	0	110S	0			12C	0
63S	0	79S	0	95C	3	111H	1			13H	1
64C	2	80S	0	96C	0	112H	0			14H	2
65C	0	81C	1	97S	1	113H	0			15H	4
66C	3	82C	2	98S	0	114H	0			16H	0

* subsurvey date; S = steer ; C = cow ; H = heifer ; B = bull

APPENDIX A

(ii) Whole animal data - detail

* 29-9-77		29-9-77		29-9-77		29-9-77		29-9-77		29-9-77	
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
17H	0	34S	0	51B	0	68C	4	86C	2	103S	0
18C	0	35S	0	52C	0	69C	0	87C	3	104S	0
19C	4	36S	0	53S	0	70C	3	88C	0	105S	0
20C	0	37S	0	54S	0	71C	2	89C	4		
21H	1	38S	0	55S	1	72C	0	90C	0		
22H	0	39C	2	56S	0	73S	0	91C	0		
23H	0	40C	0	57S	0	74H	0	92C	0		
24H	0	41H	0	58H	0	75H	2	93C	0		
25S	0	42H	0	59C	1	76H	0	94S	0		
26S	0	43H	1	60S	1	77H	0	95S	0		
27S	0	44H	0	61S	1	78H	0	96H	0		
28S	0	45H	0	62C	2	79H	0	97H	0		
29H	0	46H	0	63C	3	80C	2	98H	1		
30S	0	47H	4	64H	2	81C	2	99H	0		
31S	0	48H	0	65H	0	83C	1	100H	0		
32S	0	49H	0	66H	2	84C	4	101H	0		
33S	0	50H	0	67H	0	85C	2	102S	1		

* subsurvey date ; S = steer ; C = cow ; H = heifer ; B = bull

APPENDIX A

(ii) Whole animal data - detail

* 6-10-77		6-10-77		6-10-77		6-10-77		6-10-77		6-10-77	
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
1H	2	16C	2	31C	0	46H	1	61C	0	76S	0
2H	0	17C	1	32C	4	47H	0	62C	0	77C	3
3H	0	18C	0	33C	1	48H	0	63C	0	78C	0
4H	0	19C	0	34C	0	49H	0	64C	0	79C	0
5H	1	20C	2	35C	0	50H	1	65H	0	80H	0
6H	0	21S	0	36H	0	51H	0	66H	0	81H	0
7H	0	22S	0	37H	2	52H	2	67H	0	82H	1
8C	1	23S	0	38H	0	53H	2	68C	1	83H	0
9C	3	24S	0	39H	0	54H	0	69C	0	84C	2
10C	0	25S	0	40H	0	55S	1	70H	0	85C	2
11C	1	26S	1	41H	2	56C	4	71H	0	86C	0
12C	0	27S	0	42H	0	57C	2	72C	1	87C	1
13C	2	28S	0	43H	2	58C	1	73C	0	88C	0
14C	0	29S	0	44H	0	59H	0	74C	0	89H	0
15C	3	30C	2	45H	1	60H	0	75C	0	90H	0

* subsurvey date ; S = steer ; C = cow ; H = heifer ; B = bull

APPENDIX A

(ii) Whole animal data - detail

* 6-10-77		6-10-77		6-10-77		6-10-77					
ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS	ANIMAL	AFFECTED TEATS
91H	0	107C	0	123S	0	139C	0	155C	0		
92C	2	108C	3	124S	0	140H	0	156H	0		
93C	0	109C	2	125S	0	141H	0	157H	0		
94C	0	110C	2	126S	0	142H	0	158H	0		
95C	1	111C	0	127S	0	143H	0	159H	0		
96C	2	112C	0	128S	0	144S	1				
97C	0	113H	0	129S	0	145C	3				
98C	2	114H	0	130S	0	146S	2				
99C	3	115H	4	131S	0	147S	0				
100C	2	116H	0	132S	1	148S	0				
101C	1	117H	0	133S	0	149S	0				
102C	0	118C	4	134S	1	150S	1				
103C	0	119C	1	135S	0	151S	0				
104H	0	120C	1	136C	0	152S	0				
105H	0	121S	0	137C	0	153C	3				
106C	4	122S	2	138C	0	154S	4				

* subsurvey date ; S = steer ; C = cow ; H = heifer ; B = bull

APPENDIX A

(iii) TEAT DATA - DETAIL — SUBSURVEY OF 1/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
1	1	0	0	1
2	3	0	10	13
3	0	1	9	10
4	0	1	0	1
5	1	2	2	5
6	1	0	0	1
7	5	0	1	6
8	2	1	4	7
9	1	0	3	4
10	3	0	4	7
11	3	0	0	3
12	2	0	0	2
13	0	0	1	1
14	1	0	0	1
15	2	0	0	2
16	1	0	1	2
17	3	2	2	7
18	1	1	0	2
19	1	0	0	1
20	1	0	0	1
21	1	0	1	2
22	1	0	0	1
23	1	0	1	2
24	1	0	7	8
25	1	0	3	4
26		1	5	6
27	4	0	0	4
28	0	0	9	9
29	1	0	23	24
30	2	0	0	2
31	1	0	2	3

APPENDIX A

(iii) TEST DATA - DETAIL SUBSURVEY OF 1/9/77

NUMBER	NUMBER FROND	NUMBER [illegible] OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
33	0	0	2	2
34	1	0	0	1
35	1	1	0	2
36	0	0	6	6
37	0	1	0	1
38	1	1	1	3
39	0	0	1	1
40	0	0	2	2
41	0	0	2	2
42	0	1	3	4
43	1	1	3	5
44	0	0	1	1
45	1	1	0	2
46	0	1	6	7
47	0	1	0	1
48	0	3	0	3
49	0	0	4	4
50	0	1	2	3
51	0	1	0	1
52	0	0	2	2
53	0	0	1	1
54	1	1	0	2
55	0	0	1	2
56	0	0	5	5
57	1	1	1	2
58	0	0	0	1
59	1	1	0	2
60	1	0	0	1
61	0	0	3	4
62	0	1	1	2
		0	0	2

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 1/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
63	1	0	0	1
64	1	0	0	1
65	0	1	6	7
66	4	1	2	6
67	0	0	1	1
68	1	0	6	7
69	0	2	0	2
70	1	0	0	1
71	0	1	2	3
72	0	0	6	6
73	8	0	4	12
74	0	1	3	4
75	0	0	6	6
76	0	1	1	2
77	0	0	3	3
78	0	1	2	3
79	0	0	4	4
80	0	0	1	1
81	1	0	0	1
82	0	2	1	3
83	1	2	3	6
84	0	1	2	3
85	[illegible]	1	1	4
86	0	1	3	4
87	0	0	2	2
88	1	1	0	2
89	[illegible]	4	28	34
90	0	2	18	20
91	0	2	31	33
92	0	0	12	12
93	1	2	16	19

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 8/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
1	0	2	3	5
2	2	0	1	3
3	0	0	5	5
4	0	1	1	2
5	1	2	3	6
6	1	0	2	3
7	0	1	1	2
8	0	1	0	1
9	0	1	0	1
10	0	1	2	3
11	1	2	6	9
12	1	0	0	1
13	0	1	2	3
14	3	0	0	3
15	2	0	0	2
16	1	0	0	1
17	1	0	0	1
18	2	0	0	2
19	1	1	1	3
20	3	1	5	9
21	1	0	1	2
22	0	0	3	3
23	0	0	6	6
24	2	1	0	3
25	3	0	0	3
26	2	0	4	6
27	1	0	3	4
28	1	0	2	3
29	0	0	4	4
30	0	1	5	6
31	1	1	1	3
32	1	0	0	1

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 8/9/77

NUMBER	NUMBER FROND	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
33	2	0	0	2
34	2	1	3	6
35	2	2	0	4
36	0	2	1	3
37	1	0	4	5
38	1	1	3	5
39	1	3	12	16
40	1	2	5	8
41	10	0	0	10
42	3	0	0	3
43	7	0	0	7
44	5	1	5	11
45	0	2	15	17
46	0	1	7	8
47	0	0	6	6
48	0	1	3	4
49	.	0	0	2
50	0	2	1	3
51	2	1	9	12
52	2	1	4	7
53	1	0	0	1
54	1	0	2	3
55	2	0	0	2
56	0	2	0	2
57	1	0	1	2
58	.	0	0	2
59	1	0	4	5
60	1	0	0	1
61	1	0	0	1
62	0	0	5	5
63	0	3	4	7
64	0	1	0	1

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 8/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
65	0	0	3	3
66		0	3	3
67	0	1	2	3
68	3	0	2	5
69	1	0	1	2
70	0	2	1	3
71	2	0	6	8
72	0	1	0	1
73	0	0	1	1
74	1	1	4	6
75	0	2	6	8
76	2	0	3	5
77	4	1	4	9
78	0	2	3	5
79	0	2	10	12
80	1	1	0	2
81	1	0	0	1
82	0	2	3	5
83	1	1	4	6
84	2	1	6	9
85	0	2	0	2
86	0	1	8	9
87	0	1	5	6
88	0	1	4	5
89	0	1	1	2
90	0	0	8	8
91	0	0	6	6
92	0	2	1	3
93	0	1	6	7
94	1	0	0	1
95	0	1	0	1
96	0	0	4	4
97	0	1	0	1

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 8/9/77

NUMBER	NUMBER FROND	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
98	1	0	0	1
99	0	0	4	4
100	0	1	4	5
101	0	2	0	2
102	0	3	2	5
103	0	2	0	2
104	0	1	0	1
105	2	0	0	2
106	1	1	0	2
107	[illegible]	0	1	3
108	1	0	6	7
109	1	0	1	2
110	0	5	0	5
111	1	0	0	1
112	1	0	0	1
113	1	0	0	1
114	0	[illegible]	3	5
115	2	1	20	23
116	0	1	0	1
117	1	1	3	5
118	0	1	2	3
119	2	0	0	2
120	2	0	0	2
121	1	1	12	14
122	0	6	0	6
123	3	1	30	34
124	0	3	0	3
125	0	0	3	3
126	0	0	8	8
127	4	0	0	4
128	0	1	30	31
129	0	4	0	4

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 15/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
1	8	0	0	8
2	2	0	9	10
3	0	2	4	6
4	1	0	0	1
5	0	1	2	3
6	1	0	0	1
7	0	2	5	7
8	2	0	0	2
9	2	3	0	5
10	0	2	3	5
11	0	2	3	5
12	3	1	10	14
13	1	0	0	1
14	1	1	4	6
15	1	2	6	9
16	1	0	0	1
17	4	3	0	7
18	2	0	3	5
19	3	2	6	11
20	3	0	4	7
21	4	0	0	4
22	35	0	8	43
23	11	1	9	21
24	7	2	8	17
25	0	1	0	1
26	0	4	6	10
27	1	3	0	4
28	0	2	4	6
29	2	1	4	7
30	2	1	10	13

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 15/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
31	1	0	0	1
32	3	0	4	7
33	1	3	0	4
34	2	0	6	8
35	2	1	0	3
36	1	1	1	3
37	1	0	0	1
38	2	2	3	5
39	0	1	0	1
40	1	1	3	5
41	0	4	0	4
42	0	0	1	1
43	2	0	0	2
44	0	2	4	6
45	1	0	2	3
46	1	0	2	3
47	0	1	4	5
48	0	0	6	6
49	0	1	3	4
50	2	0	1	3
51	1	0	1	2
52	2	0	0	2
53	1	1	0	2
54	0	1	1	2
55	0	4	0	4
56	1	2	0	3
57	1	0	0	1
58	1	2	0	3
59	0	1	1	2
60	0	0	3	3
61	0	0	2	2
62	0	2	0	2

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 15/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
63	1	1	0	2
64	0	0	2	2
65	1	1	5	7
66	1	0	0	1
67	1	0	1	2
68	1	0	0	1
69	0	1	1	2
70	0	4	2	6
71	0	0	2	2
72	1	0	0	1
73	2	6	1	9
74	1	0	0	1
75	0	0	1	1
76	0	1	0	1
77	1	0	1	2
78	1	0	4	5
79	1	0	0	1
80	0	4	0	4
81	0	4	2	6
82	18	0	2	20
83	11	0	0	11

APPENDIX A

(iii) TEAT DATA - DETAIL SUBSURVEY OF 29/9/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
1	1	3	4	8
2	1	4	15	20
3	2	0	4	6
4	0	1	1	2
5	.	2	1	5
6	1	3	4	8
7	0	1	2	3
8	0	0	6	6
9	0	2	1	3
10	0	0	1	1
11	1	0	2	3
12	0	0	5	5
13	1	0	0	1
14	1	0	1	2
15	1	1	1	3
16	1	0	0	1
17	0	.	3	5
18	2	0	0	2
19	2	0	0	2
20	2	1	3	6
21	0	2	2	4
22	1	2	1	4
23		0	1	3
24	2	1	1	4
25	0	0	1	1
26	1	0	1	2
27	.	0	1	3
28	0	0	1	1
29	1	1	0	2
30		1	3	6
31	1	0	0	1
32	2	0	0	2
33	0	4	1	5

APPENDIX A

(iii) TEST DATA - DETAIL SUBSURVEY OF 29/9/77

NUMBER	NUMBER PRONO	NUMBER PLANT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
34	0	1	2	3
35	0	1	2	3
36	[illegible]	[illegible]	4	8
37	0	1	2	3
38	[illegible]	0	0	2
39	0	1	0	1
40	[illegible]	10	15	27
41	0	0	1	1
42	1	0	0	1
43	0	[illegible]	4	6
44	1	4	9	17
45	0	[illegible]	1	4
46	1	0	0	1
47	0	[illegible]	0	[illegible]
48	0	[illegible]	4	6
49	[illegible]	0	0	2
50	0	1	0	1
51	0	1	0	1
52	12	0	18	30
53	27	0	12	39
54	17	0	22	39
55	23	0	8	31
56	4	4	8	16
57	0	4	5	9
58	0	4	2	6
59	0	14	6	20
60	0	4	6	10
61	1	2	3	6
62	0	6	2	8
63	0	1	4	5
64	0	3	1	4
65	[illegible]	0	8	13
66	[illegible]	0	8	14
67	8	[illegible]	4	14

APPENDIX A

(iii) TEST DATA - DETAIL SUBSURVEY OF 6/10/77

NUMBER	NUMBER FROM	NUMBER [illegible] OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
1	1	1	1	3
2	1	0	0	1
3	5	0	0	5
4	0	2	0	2
5		1	0	6
6	1	0	0	1
7	0	2	0	2
8		1	0	3
9	3	0	0	3
10	0	6	0	6
11	0	3	0	3
12	0	9	0	9
13	0	3	0	3
14		0	1	3
15	0	3	0	3
16	1	0	9	10
17	4	0	1	5
18	1	0	0	1
19	1	0	0	1
20	1	0	0	1
21	1	3	0	4
22	0	3	0	3
23	1	2	0	3
24	0	2	3	5
25	2	0	0	2
26	1	0	0	1
27	1	0	1	2
28	3	4	4	11
29	0	3	2	5
30	2	0	2	4
31	1	0	2	3
32		0	3	5
33	1	0	0	1

APPENDIX A

(iii) TEAT DATA - DETAIL — SUBSURVEY OF 6/10/77

NUMBER	NUMBER FRONT	NUMBER FLAT OR ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
34	1	0	20	21
35	4	0	12	16
36		4	0	5
37	5	.	3	8
38	1	0	0	1
39	2	0	0	2
40	1	0	0	1
41	0	4	1	5
42	0	4	8	12
43	0	0	3	3
44	.	0	0	2
45	1	.	1	4
46	4	0	0	4
47	0	0	1	1
48	0	.	0	2
49	1	0	0	1
50	1	0	8	9
51	2	0	3	5
52	2	0	4	6
53	0	4	6	10
54	0	0	10	10
55	1	1	2	4
56	4	0	3	7
57	0	0	2	2
58	1	0	1	2
59	1	0	0	1
60	1	0	0	1
61	1	0	0	1
62	0	0	1	1
63	0	.	0	2
64	0	'	0	2
65	0	1	0	1
66	0	"	3	9

APPENDIX A

(iii) TEST DATA - DETAIL SUBSURVEY OF 6/10/77

NUMBER	NUMBER FRONT	NUMBER FLAT ON ROUND	NUMBER RICE GRAIN	TOTAL NUMBER LESIONS
67	1	0	0	1
68	0	1	4	5
69	0	0	13	13
70	0	5	0	5
71	2	0	0	2
72	0	3	0	3
73	3	0	0	3
74	1	0	0	1
75	1	0	0	1
76	3	0	1	4
77	1	2	3	6
78	0	2	4	6
79	1	1	6	8
80	1	0	0	1
81	1	0	0	1
82	0	1	0	1
83	0	1	0	1
84	0	1	0	1
85	2	0	0	2
86	1	0	0	1
87	1	0	0	1
88	1	0	0	1
89	0	2	6	8
90	2	3	6	11
91	1	0	3	4
92	1	0	0	1
93	1	0	0	1
94	3	0	3	6
95	2	2	0	4
96	0	2	2	4
97	0	2	0	2
98	0	1	0	1

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM R G A (Test Rice Grain - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.8	4.6	4.7	23	4.6	4.0	4.3
2	4.5	4.3	4.4	24	4.2	4.2	4.2
3	4.1	3.9	4.0	25	4.3	4.4	4.35
4	4.6	4.0	4.3				
5	4.2	4.0	4.1				
6	4.0	3.8	3.9				
7	3.6	4.0	3.8				
8	3.8	4.	4.0				
9	3.8	4.4	4.1				
10	3.8	3.8	3.8				
11	3.9	4.5	4.2				
12	3.8	4.2	4.0				
13	3.6	4.0	3.8				
14	3.6	3.8	3.7				
15	3.8	3.9	3.85				
16	3.9	4.0	3.95				
17	4.5	4.3	4.4				
18	4.5	4.	4.35				
19	3.6	3.9	3.75				
20	4.	4.0	4.2				
21	3.8	4.0	3.9				
22	3.8	4.0	3.9				

A and B = two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of RPV particle diameters.

EXTRACT FROM R G B (Test Rice Grain - Single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.0	4.3	4.15	23	4.1	4.1	4.1
2	4.0	3.9	3.95	24	3.9	4.5	4.2
3	3.75	4.1	3.93	25	3.7	4.1	3.9
4	3.8	3.9	3.85	26	4.0	4.1	4.05
5	3.9	3.9	3.9	27	4.2	3.8	4.00
6	4.0	3.8	3.9	28	4.1	4.0	4.05
7	4.1	4.0	4.15	29	4.0	3.8	3.9
8	3.8	3.9	3.85	30	4.0	4.0	4.0
9	3.6	3.8	3.7	31	4.0	4.0	4.0
10	4.0	3.9	3.95	32	4.1	3.9	4.0
11	3.9	4.0	3.95	33	4.0	3.9	3.95
12	4.1	4.0	4.1	34	3.8	4.0	3.90
13	4.2	4.1	4.15	35	4.2	3.9	4.05
14	4.6	4.0	4.3	36	3.9	4.0	3.95
15	4.4	4.1	4.25	37	4.0	3.8	3.9
16	4.3	4.2	4.25	38	4.1	3.9	4.0
17	4.2	4.1	4.15	39	3.8	4.2	4.0
18	4.1	3.9	4.0	40	4.0	3.8	3.9
19	4.1	4.1	4.1	41	3.6	3.9	3.75
20	4.0	4.2	4.1	42	3.9	3.8	3.85
21	4.1	4.3	4.2	43	3.9	3.8	3.85
22	3.9	4.0	3.95	44	4.0	4.1	4.05

A and B = two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of RPV particle diameters.

EXTRACT FROM R G B (Teat Rice Grain - Single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	3.9	3.9	3.9	23	3.9	3.9	3.9
2	3.8	4.[illegible]	4.0	24	4.0	3.8	3.9
3	4.0	4.0	4.0	25	3.8	4.2	4.0
4	4.[illegible]	4.[illegible]	4.15	26	3.9	4.1	4.0
5	3.9	3.9	3.9	27	4.2	4.5	4.35
6	3.8	4.0	3.9	28	4.2	4.1	4.15
7	4.9	5.0	4.95	29	4.5	4.8	4.65
8	4.[illegible]	4.[illegible]	4.[illegible]	30	4.2	4.1	4.15
9	4.[illegible]	4.[illegible]	4.55	31	4.7	4.5	4.6
10	4.[illegible]	4.[illegible]	4.[illegible]	32	3.9	4.0	3.95
11	4.0	4.1	4.05	33	4.0	3.9	3.95
12	4.[illegible]	4.[illegible]	4.[illegible]	34	3.8	3.8	3.8
13	4.9	5.0	4.95	35	3.9	4.0	3.95
14	5.0	3.8	4.4	36	3.8	4.0	3.9
15	4.8	4.[illegible]	4.55	37	3.7	3.9	3.8
16	4.[illegible]	4.[illegible]	4.[illegible]	38	3.9	3.9	3.9
17	4.1	4.[illegible]	4.[illegible]	39	3.9	3.7	3.8
18	4.[illegible]	4.[illegible]	4.4	40	4.0	3.9	3.95
19	4.1	4.1	4.1	41	3.9	3.9	3.9
20	4.[illegible]	4.[illegible]	4.[illegible]	42	3.7	4.0	3.85
21	4.[illegible]	4.1	4.3	43	3.9	4.0	3.95
22	4.0	3.7	3.85	44	4.0	4.0	4.0

A and B = two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of B/V particle diameters.

EXTRACT FROM A C B (Test Rice Grain - Single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	3.9	4.0	3.95	23	3.8	3.9	3.85
2	4.1	3.8	4.0	24	3.8	3.9	3.85
3	4.1	4.0	4.1	25	3.8	3.6	3.7
4	4.0	3.8	3.9	26	3.8	3.9	3.85
5	3.7	3.8	3.75	27	4.5	4.0	4.25
6	3.9	4.0	3.95	28	3.9	4.0	3.95
7	3.9	4.0	3.95	29	3.9	3.9	3.9
8	3.8	4.1	3.95	30	3.8	3.9	3.85
9	3.8	3.9	3.85	31	3.8	3.8	3.8
10	4.0	4.1	4.05	32	3.8	3.9	3.85
11	3.9	4.1	4.0	33	3.7	3.8	3.75
12	3.8	4.0	3.9	34	3.8	3.9	3.85
13	3.9	4.1	4.05	35	3.8	4.1	3.95
14	3.8	4.0	3.9	36	4.0	3.9	3.95
15	3.9	4.3	4.1	37	3.9	3.9	3.9
16	3.9	3.8	3.85	38	4.3	4.7	4.5
17	3.8	3.9	3.85	39	4.5	4.2	4.35
18	3.8	3.8	3.8	40	4.2	4.5	4.35
19	3.9	3.8	3.85	41	4.5	4.0	4.25
20	4.1	3.9	4.05	42	4.5	4.2	4.35
21	4.0	4.0	4.0	43	4.0	3.9	3.95
22	3.8	4.0	3.9	44	4.5	4.3	4.4

A and B two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of SPV particle diameters.

EXTRACT FROM R G B (Test Rice Grain - Single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.0	4.0	4.0	23	4.1	3.8	3.95
2	4.1	4.1	4.1	24	4.0	3.6	3.8
3	4.0	4.1	4.1	25	4.5	3.8	4.15
4	4.1	4.0	4.05	26	4.0	3.8	3.9
5	3.8	3.9	3.85	27	4.3	3.9	4.1
6	4.0	4.0	4.1	28	4.1	4.0	4.05
7	4.0	4.1	4.05	29	3.9	4.2	4.05
8	3.9	4.0	3.95	30	3.9	4.0	3.95
9	3.9	3.8	3.7	31	4.1	3.9	4.0
10	3.9	3.8	3.7	32	4.1	4.0	4.05
11	3.8	3.9	3.85	33	4.3	4.2	4.25
12	4.0	3.8	3.9	34	3.9	3.8	3.85
13	3.8	3.8	3.8	35	4.0	3.9	3.95
14	3.9	3.8	3.85	36	4.0	3.8	3.9
15	3.9	4.0	3.95	37	4.0	4.0	4.0
16	3.8	3.6	3.7	38	4.0	3.9	3.95
17	3.8	4.0	3.9	39	4.0	4.2	4.1
18	3.9	3.8	3.7	40	3.8	4.1	3.95
19	4.1	3.9	4.0	41	3.9	3.9	3.9
20	3.9	3.7	3.8	42	3.8	4.2	4.0
21	4.2	3.8	4.0	43	4.5	3.7	4.1
22	4.2	3.8	4.0	44	3.8	3.9	3.85

A and B - two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM R G B (Teat Rice Grain - Single case)

Particle Number	Measurement(mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.1	4.0	4.1	23	3.9	3.9	3.9
2	4.0	3.9	3.9	24	4.1	3.9	4.0
3	4.0	4.1	4.0				
4	4.1	4.1	4.1				
5	3.9	3.5	3.7				
6	4.0	4.1	4.0				
7	4.1	4.1	4.1				
8	3.9	3.8	3.8				
9	3.8	4.0	3.9				
10	3.9	3.9	3.9				
11	3.9	4.0	3.9				
12	4.0	3.9	3.9				
13	3.9	3.9	3.9				
14	4.1	3.8	4.0				
15	4.0	3.8	3.9				
16	4.2	4.1	4.1				
17	4.1	4.0	4.0				
18	4.1	4.0	4.0				
19	3.9	4.1	4.0				
20	3.9	3.8	3.8				
21	3.7	3.8	3.7				
22	4.1	4.1	4.1				

A and B - two diameter measurements made at 90^{0} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM R G C (Test Rice Grain - pooled)

Particle Number	Measurement(mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	5.0	5.2	5.1	26	4.8	4.9	4.85
2	4.9	5.1	5.05	24	4.9	5.2	5.05
3	5.0	5.2	5.1	25	5.1	5.0	5.05
4	5.0	4.6	4.8	26	5.0	4.6	4.8
5	5.0	5.2	5.1	27	5.0	4.7	4.85
6	5.0	4.7	4.85	28	4.8	4.8	4.8
7	5.0	5.1	5.05	29	5.1	5.2	5.15
8	4.8	5.5	5.15	30	5.1	5.1	5.1
9	4.9	5.1	5.0	31	5.2	5.3	5.25
10	5.0	5.2	5.1	32	5.1	5.3	5.2
11	4.7	4.9	4.8	33	5.0	5.1	5.05
12	5.5	5.0	5.15	34	5.5	5.0	5.25
13	5.0	5.1	5.05	35	5.2	5.6	5.4
14	5.2	5.2	5.2	36	4.8	4.9	4.85
15	4.9	5.0	4.95	37	4.8	4.9	4.85
16	4.9	5.0	4.95	38	4.7	5.0	4.85
17	4.8	5.2	5.0	39	4.5	4.6	4.55
18	5.5	5.0	5.25	40	4.7	4.8	4.75
19	4.8	5.0	4.9	41	4.6	5.2	4.9
20	4.8	4.9	4.85	42	4.9	5.1	5.0
21	4.7	5.0	4.85	43	5.5	4.7	5.1
22	4.5	5.0	4.75	44	4.8	5.6	5.2

A and B - two diameter measurements made at 90^{o} to each other

APPENDIX B - Measurement of NPV particle diameters.

EXTRACT FROM R & C (Test Rice Grain - pooled)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.9	5.1	5.0	23	5.0	5.0	5.0
2	5.0	5.2	5.1	24	5.3	5.0	5.15
3	5.0	5.1	5.05	25	4.7	4.9	4.8
4	5.1	5.3	5.2	26	5.0	4.9	4.95
5	5.0	5.3	5.15	27	4.9	5.1	5.0
6	4.9	5.1	5.0	28	5.1	4.7	4.9
7	5.1	5.2	5.2	29	4.9	4.8	4.85
8	4.7	5.7	5.2	30	4.5	4.8	4.65
9	5.3	5.8	5.05	31	4.9	5.2	5.05
10	5.2	5.3	5.25	32	5.0	5.1	5.05
11	5.3	5.5	5.4	33	5.0	5.2	5.1
12	4.9	5.1	5.2	34	5.5	5.1	5.3
13	4.8	4.7	4.75	35	4.8	5.0	4.9
14	4.8	5.1	4.95	36	5.1	5.5	5.3
15	4.8	4.8	4.8	37	4.7	5.0	4.85
16	4.7	5.9	4.8	38	5.0	5.2	5.1
17	5.0	5.2	5.1	39	5.1	5.2	5.15
18	4.9	5.1	4.95	40	4.9	5.3	5.1
19	5.0	4.9	4.95	41	5.0	5.2	5.1
20	4.9	5.1	5.0	42	4.9	5.2	5.05
21	5.1	5.3	5.2	43	5.0	5.1	5.05
22	4.9	5.0	4.95	44	5.2	5.2	5.2

A and B - two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of bPV particle diameters.

EXTRACT FROM R C C (Test Rice Grain - pooled)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	5.8	5.0	4.9				
2	4.9	5.3	5.1				
3	4.8	5.1	4.85				
4	4.7	4.8	4.75				
5	4.9	5.0	4.95				
6	4.9	5.3	5.1				
7	5.2	5.0	5.1				
8	4.9	5.1	5.0				
9	5.0	4.7	4.85				
10	4.9	5.0	4.95				
11	4.9	5.1	5.0				
12	4.7	5.2	4.95				

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM FA (Test front - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	5.0	5.5	5.25	23	5.0	4.8	4.9
2	4.9	5.1	5.05	24	4.5	4.9	4.7
3	4.9	5.5	4.7	25	4.8	5.5	5.15
4	4.8	4.8	4.8	26	5.0	5.3	5.15
5	4.5	4.7	4.6	27	5.0	5.2	5.1
6	4.5	5.1	4.85	28	4.8	4.8	4.8
7	4.4	4.7	4.65	29	4.2	4.9	4.55
8	4.8	4.7	4.75	30	4.8	4.3	4.55
9	4.4	4.9	4.7	31	4.3	4.7	4.5
10	4.4	4.8	4.65	32	4.6	5.0	4.8
11	4.5	5.1	5.0	33	4.2	4.8	4.5
12	5.0	5.2	5.1	34	5.2	5.0	5.1
13	5.0	4.5	4.75	35	4.9	5.0	4.95
14	5.0	4.9	5.1	36	5.0	4.9	4.95
15	5.1	5.0	5.1	37	5.0	4.9	4.95
16	5.0	5.1	5.1	38	5.2	4.9	5.05
17	5.0	5.0	5.0	39	5.3	5.0	5.15
18	5.0	4.9	4.95	40	5.0	5.1	5.05
19	4.8	5.0	4.9	41	5.2	5.9	5.55
20	4.8	4.8	4.8	42	4.9	5.0	4.95
21	4.9	5.0	4.95	43	5.1	5.0	5.05
22	4.8	5.0	4.9	44	5.2	5.0	5.1

A and B = two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of HPV particle diameters.

EXTRACT FROM FA (Test frond single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	5.0	5.8	5.4				
2	4.8	5.0	4.9				
3	5.0	5.2	5.1				
4	5.2	5.1	5.15				
5	4.8	4.9	4.85				
6	5.0	5.3	5.15				

A and B - two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM FB - (single case test frond)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.[illegible]	4.5	4.55	23	4.5	4.6	4.55
2	4.1	3.9	4.0	24	4.6	4.0	4.3
3	4.5	4.4	4.45	25	4.2	5.0	4.6
4	4.[illegible]	4.5	4.4	26	4.8	4.9	4.85
5	5.0	5.0	5.0	27	5.0	5.0	5.0
6	5.0	4.9	4.95	28	4.8	4.7	4.75
7	4.8	4.[illegible]	4.65	29	4.8	4.5	4.65
8	5.0	4.8	4.9	30	4.5	4.6	4.55
9	4.[illegible]	4.8	4.5	31	5.0	5.2	5.1
10	5.[illegible]	4.[illegible]	4.0	32	4.8	5.3	5.05
11	4.	4.0	4.1	33	4.8	5.0	4.9
12	4.	4.[illegible]	4.[illegible]	34	5.0	5.0	5.0
13	4.8	4.7	4.75	35	5.1	5.0	5.05
14	4.9	5.1	5.05	36	4.5	4.8	4.65
15	5.[illegible]	4.5	4.85	37	4.7	4.8	4.75
16	4.[illegible]	4.9	4.6	38	4.5	4.7	4.6
17	4.9	4.3	4.6	39	5.2	5.5	5.35
18	4.0	5.0	4.5	40	5.3	4.9	5.1
19	4.6	4.3	4.45	41	5.5	5.0	5.25
20	4.[illegible]	4.[illegible]	4.35	42	4.8	4.8	4.8
21	4.7	4.[illegible]	4.5	43	5.0	5.2	5.1
22	4.[illegible]	5.0	4.6	44	5.5	5.5	5.5

A and B - two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of B/V particle diameters.

EXTRACT FROM FB (Single case test trend)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.8	.8	5.3	23	5.2	5.5	5.35
2	5.1	4.5	4.8	24	5.5	4.5	5.0
3	5.0	4.8	4.9	25	5.8	4.9	5.35
4	5.0	5.2	5.1	26	4.8	5.2	5.0
5	4.8	.0	4.9	27	4.8	5.2	5.0
6	5.0	4.8	4.9	28	5.2	5.1	5.15
7	5.	..	5.	29	4.6	5.5	5.05
8	5.0	5.	5.1	30	5.0	5.3	5.15
9	4.9	1.8	4.85	31	4.8	5.2	5.0
10	4.4	4.9	4.75	32	4.7	4.5	4.6
11	4.9	4.7	4.8	33	4.6	4.9	4.75
12	4.3	4.4	1.5	34	4.5	5.1	4.8
13	5.1	5.2	5.15	35	4.5	4.0	4.25
14	5.0	5.	5.1	36	4.8	4.9	4.85
15	5.3	5.3	5.3	37	4.5	4.9	4.7
16	4.9	.3	5.1	38	4.8	5.0	4.9
17	5.	..	5.35	39	4.8	4.9	4.85
18	5..	4.5	4.85	40	4.5	4.8	4.65
19	5.7	4.7	5.2	41	4.8	4.8	4.8
20	4.8	.0	4.9	42	4.5	4.8	4.65
21	4.5	5.2	1.85	43	4.8	4.9	4.85
22	4.4	5.2	4.9	44	5.0	5.0	5.0

A and B two diameter measurements made at 90^{0} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM FB (Single case test found)

Particle Number	Measurement(mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.8	5.0	4.9	23	4.5	4.8	4.65
2	4.7	5.0	4.85	24	4.9	4.8	4.85
3	5.1	5.0	5.15	25	5.3	4.9	5.1
4	4.7	4.7	4.7	26	5.3	5.0	5.15
5	4.8	5.0	4.75	27	4.7	4.9	4.8
6	4.7	5.1	4.9	28	4.5	4.9	4.7
7	5.2	5.0	5.1	29	4.8	5.0	4.9
8	5.5	4.8	5.0	30	4.9	4.9	4.9
9	4.7	5.3	5.0	31	4.8	5.0	4.9
10	4.6	4.9	4.75	32	5.0	5.0	5.0
11	4.5	4.7	4.6	33	4.7	4.9	4.8
12	4.7	5.1	4.95	34	5.1	5.3	5.2
13	4.8	5.0	4.9	35	4.8	5.0	4.9
14	4.6	4.9	4.7	36	4.8	4.9	4.85
15	4.7	5.0	4.85	37	4.8	5.0	4.9
16	4.9	5.3	5.1				
17	4.8	5.2	5.0				
18	4.9	5.3	5.1				
19	4.8	4.8	4.8				
20	4.7	4.9	4.8				
21	4.8	4.8	4.8				
22	4.8	4.9	4.85				

A and B two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM FC (Test front - pooled sample)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.8	5.0	4.9	23	4.8	5.4	5.1
2	4.8	4.9	4.85	24	4.8	5.5	5.1
3	5.3	4.8	5.0	25	4.8	5.0	4.9
4	4.9	5.1	5.0	26	5.1	5.3	5.2
5	5.0	5.0	5.0	27	4.8	4.9	4.85
6	4.8	5.5	5.15	28	4.6	4.9	4.75
7	5.0	5.2	5.1	29	4.5	5.8	5.25
8	4.8	5.0	4.9	30	5.0	4.6	4.8
9	4.9	5.0	4.95	31	5.0	5.0	5.0
10	4.6	5.0	4.8	32	4.8	6.0	5.4
11	4.8	4.5	4.65	33	5.5	5.2	5.35
12	4.4	5.0	4.7	34	5.0	5.2	5.1
13	4.5	4.1	4.3	35	6.0	6.0	6.0
14	4.8	5.0	4.9	36	4.5	5.0	4.75
15	4.8	5.1	4.95	37	5.0	5.0	5.0
16	4.8	4.8	4.8	38	5.0	5.2	5.1
17	5.0	5.1	5.1	39	5.0	5.7	5.35
18	4.7	4.9	4.95	40	5.3	5.2	5.25
19	4.8	4.9	4.85	41	5.0	5.4	5.2
20	4.5	4.7	4.6	42	4.5	5.2	4.85
21	4.7	4.2	4.4	43	4.9	5.0	4.95
22	4.6	5.0	4.8	44	4.9	5.0	4.95

A and B - two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of RPV particle diameters.

EXTRACT FROM E 8 C (Test frond, pooled sample)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.9	5.1	5.0	23	4.8	5.0	4.9
2	5.0	5.3	5.15	24	4.0	4.5	4.25
3	4.5	5.0	4.75	25	4.5	4.9	4.7
4	4.5	5.3	4.9	26	4.0	4.8	4.4
5	4.7	4.8	4.75	27	4.5	5.6	5.05
6	4.8	5.3	5.05	28	4.0	4.0	4.0
7	4.5	5.2	4.85	29	4.2	4.5	4.35
8	4.2	4.8	4.55	30	4.2	4.6	4.4
9	4.5	5.0	4.75	31	4.2	4.9	4.55
10	4.5	5.0	4.75				
11	5.1	4.8	4.95				
12	4.8	4.8	4.8				
13	4.5	5.2	4.85				
14	4.5	4.9	4.7				
15	4.8	4.5	4.75				
16	5.0	4.7	4.85				
17	5.4	5.2	5.15				
18	4.5	5.0	4.75				
19	4.3	5.0	4.65				
20	4.5	5.2	4.85				
21	4.7	5.2	4.95				
22	4.7	5.6	5.15				

A and B - two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of BW particle diameters.

EXTRACT FROM P 3 (pooled sample, heat flat and round)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	5.0	4.8	4.9	25	4.6	4.8	4.7
2	4.9	4.9	4.75	24	4.8	4.5	4.65
3	4.8	5.1	4.95	25	5.0	4.9	4.95
4	4.9	4.6	4.75	26	3.9	3.9	3.9
5	5.0	4.8	4.9	27	3.9	3.9	3.9
6	4.7	4.8	4.75	28	3.8	4.0	3.9
7	4.9	5.0	4.75	29	3.7	4.2	3.95
8	5.2	4.5	4.85	30	3.9	4.1	4.0
9	5.0	4.9	4.95	31	3.9	4.4	4.15
10	5.1	4.8	4.95	32	4.5	4.0	4.25
11	5.0	4.9	4.95	33	4.3	3.9	4.1
12	5.0	4.8	4.9	34	4.1	3.7	3.9
13	4.6	4.8	4.7	35	4.1	4.3	4.2
14	4.8	4.6	4.7	36	4.0	4.1	4.05
15	5.0	4.9	4.95	37	4.0	4.2	4.1
16	5.0	4.7	4.85	38	4.3	4.2	4.25
17	5.0	4.9	4.95	39	4.3	4.2	4.25
18	4.8	4.9	4.85	40	3.8	4.2	4.0
19	5.0	4.9	4.95	41	4.1	4.2	4.15
20	5.0	4.6	4.8	42	3.8	4.0	3.9
21	5.1	4.8	4.95	43	3.9	4.1	4.0
22	4.5	4.7	4.6	44	4.1	4.2	4.15

A and B - two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM P R (pocket sample boat flat and round)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	3.9	4.0	3.95	23	4.5	5.0	4.75
2	4.0	4.0	4.0	24	4.5	5.3	4.9
3	3.9	3.8	3.85	25	4.5	4.9	4.7
4	3.9	4.3	4.1	26	4.7	4.8	4.75
5	3.9	4.	4.0	27	4.9	4.9	4.9
6	4.1	4.0	4.05	28	4.0	4.5	4.25
7	5.0	.0	5.0	29	4.3	4.5	4.4
8	4.3	4.9	4.6	30	5.0	4.2	4.6
9	4.	4.0	4.75	31	4.0	5.2	4.6
10	4.2	5.	4.7	32	4.1	4.2	4.15
11	4.0	4.8	4.4	33	4.5	4.2	4.35
12	4.2	5.0	4.6	34	4.2	5.0	4.6
13	4.0	4.0	4.5	35	4.5	4.0	4.25
14	3.9	4.9	4.4	36	4.3	4.8	4.55
15	4.2	5.5	4.9	37	4.5	4.7	4.6
16	4.6	5.	4.95	38	4.0	4.9	4.45
17	4.5	5.	4.85	39	4.7	4.8	4.75
18	4.3	4.5	4.4	40	4.5	4.8	4.65
19	4.4	5.2	4.8	41	4.7	5.2	4.95
20	4.1	4.2	4.15	42	4.2	4.7	4.45
21	4.5	4.9	4.7	43	4.6	4.6	4.6
22	4.5	4.7	4.6	44	4.3	5.1	4.7

A and B - two diameter measurements made at 90^{0} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM F R (pooled sample, test flat and round)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.7	4.8	4.75	23	4.5	5.0	4.75
2	4.2	4.8	4.5	24	4.8	5.2	5.0
3	4.5	4.0	4.25	25	4.5	4.9	4.7
4	4.2	5.2	4.7	26	4.5	4.8	4.65
5	4.2	4.8	4.5	27	5.0	5.2	5.1
6	4.0	4.9	4.45	28	4.8	5.0	4.9
7	4.0	4.8	4.4	29	5.2	5.0	5.1
8	4.2	5.6	4.9	30	4.8	4.8	4.8
9	4.6	4.9	4.75	31	4.8	4.8	4.8
10	4.5	5.2	4.85	32	5.0	5.0	5.0
11	4.5	[illegible]	4.8	33	4.9	5.0	4.95
12	4.5	5.2	4.85	34	5.3	4.8	5.05
13	4.8	5.0	4.9	35	4.7	4.8	4.75
14	4.1	5.1	5.0	36	4.9	5.2	5.05
15	4.8	5.0	5.4	37	4.5	5.0	4.75
16	4.8	[illegible]	5.15	38	4.5	4.5	4.5
17	4.9	5.2	5.05	39	4.5	4.4	4.45
18	4.9	5.3	5.1	40	4.0	4.6	4.3
19	4.5	4.8	4.65	41	4.3	4.5	4.4
20	4.8	5.2	5.0	42	3.9	4.1	4.0
21	4.8	5.2	5.0	43	4.1	4.5	4.3
22	4.5	4.8	4.65	44	4.5	4.3	4.4

A and B - two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM F R (pooled sample - test flat and round)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.0	4.5	4.25	23	4.0	4.8	4.4
2	4.4	4.5	4.45	24	4.3	4.7	4.5
3	3.9	4.0	3.95	25	4.0	4.8	4.4
4	4.3	4.6	4.45	26	4.3	3.9	4.1
5	4.3	4.8	4.55	27	4.3	4.7	4.5
6	3.8	3.9	3.85	28	4.7	5.1	4.9
7	4.0	4.3	4.15	29	4.6	5.0	4.8
8	3.9	4.1	4.0	30	4.0	4.5	4.25
9	4.3	4.7	4.6	31	4.0	4.8	4.4
10	4.0	4.3	4.15	32	4.0	4.0	4.0
11	4.5	4.6	4.55	33	4.2	4.2	4.2
12	4.0	4.	4.1	34	4.5	4.5	4.5
13	3.9	4.1	4.05	35	4.2	4.5	4.35
14	4.0	4.3	4.15	36	4.2	4.0	4.1
15	4.9	4.3	4.3	37	4.1	4.5	4.3
16	3.8	4.2	4.0	38	4.5	4.5	4.5
17	4.3	4.2	4.25	39	4.7	4.9	4.8
18	4.5	4.6	4.55	40	4.6	4.2	4.4
19	4.6	4.3	4.45	41	4.0	4.8	4.0
20	4.8	4.3	4.55	42	4.8	4.6	4.7
21	4.6	4.3	4.55	43	4.0	3.9	3.95
22	4.5	5.1	4.8				

A and B = two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of CBPV particle diameters.

EXTRACT FROM CBPV 1A (cutaneous fibropapilloma - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.8	5.2	5.0	23	3.9	4.2	4.05
2	5.1	5.1	5.6	24	4.5	4.8	4.65
3	4.8	5.5	5.15	25	4.2	4.5	4.35
4	4.7	4.9	4.8				
5	4.3	4.2	4.25				
6	4.5	4.3	4.35				
7	4.3	3.9	4.1				
8	4.0	4.	4.1				
9	4.3	4.1	4.15				
10	4.5	4.1	4.3				
11	4.0	4.2	4.15				
12	4.0	5.8	5.9				
13	4.0	4.1	4.05				
14	4.3	4.5	4.35				
15	4.2	4.0	4.1				
16	4.0	4.1	4.05				
17	3.7	4.1	3.95				
18	4.8	5.2	5.0				
19	4.8	4.8	4.8				
20	4.8	5.2	5.0				
21	3.9	4.3	4.2				
22	4.0	4.7	4.35				

A and B - two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of PPV particle diameters.

EXTRACT FROM CRPV 14 (cutaneous fibropapilloma - single case)

Particle Number	Measurement(mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.8	4.8	4.8	35	4.2	4.8	4.5
2	4.2	4.6	4.4	36	4.7	4.7	4.7
3	4.0	4.1	4.05	37	5.0	4.8	4.9
4	4.2	4.8	4.5	38	3.8	4.1	3.95
5	4.5	4.7	4.6	39	4.2	4.2	4.2
6	4.2	4.8	4.15	40	3.8	4.2	4.0
7	4.8	4.9	4.85	41	3.8	3.9	3.85
8	5.2	4.8	5.	42	4.2	4.5	4.35
9	4.6	4.9	4.7	43	4.1	4.8	4.45
10	4.5	4.8	4.85	44	3.8	5.0	4.4
11	5.0	5.2	5.1	45	3.8	4.8	4.3
12	4.8	4.	4.0	46	4.0	4.6	4.3
13	4.8	5.0	4.9	47	4.2	4.6	4.4
14	4.8	4.4	4.8	48	4.5	4.7	4.6
15	4.8	4.8	4.8	49	3.9	4.8	4.35
16	3.8	4.	4.15	50	4.5	3.5	4.0
17	4.	4.	4.3	51	4.0	4.7	4.35
18	4.5	4.4	4.45	40	3.8	4.5	4.15
19	4.0	4.3	4.15	41	3.8	4.5	4.15
20	4.5	4.2	4.3	42	4.2	4.5	4.35
21	4.3	5.0	4.65	43	4.5	4.6	4.55
22	4.1	4.2	4.3	44	4.5	4.7	4.6

A and B - two diameter measurements made at 90^{0} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM CBPV 1B (cutaneous fibropapilloma - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.5	4.8	[illegible]	23	4.6	4.9	4.75
2	4.0	[illegible]	[illegible]	24	5.2	5.7	4.45
3	4.0	[illegible]	4.1	25	4.5	5.[illegible]	4.35
4	4.0	[illegible]	[illegible]	26	5.0	5.5	5.25
5	4.5	[illegible]	[illegible]	27	4.9	5.0	4.95
6	4.5	5.0	[illegible]	28	5.2	5.5	5.35
7	[illegible]	[illegible]	[illegible]	29	5.[illegible]	5.5	5.35
8	[illegible].0	[illegible].0	[illegible].0	30	5.5	5.0	5.25
9	4.8	[illegible]	[illegible].0	31	5.2	5.3	5.25
10	4.8	[illegible]	[illegible].05				
11	[illegible].5	[illegible].5	[illegible].4				
12	4.7	[illegible]	5.1				
13	4.7	4.8	[illegible]				
14	4.0	[illegible]	[illegible]				
15	4.6	[illegible]	[illegible]				
16	4.	[illegible]	[illegible]				
17	4.7	4.9	4.8				
18	[illegible]	4.8	[illegible]				
19	[illegible]	4.8	4.9				
20	4.0	[illegible]	4.25				
21	4.5	4.8	[illegible]				
22	4.7	[illegible].0	[illegible]				

A and B = two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of CPV particle diameters.

EXTRACT FROM CRPV 2 (cutaneous fibropapilloma - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.5	4.7	4.6	24	4.8	5.0	4.9
2	4.0	4.2	4.1	25	4.8	5.2	5.0
3	4.5	4.6	4.55	26	4.6	4.9	4.75
4	4.6	4.4	4.5	27	5.1	4.2	4.65
5	4.5	5.0	4.75	28	5.1	4.2	4.65
6	4.7	4.9	4.80	29	5.0	4.9	4.95
7	4.5	5.0	4.75	30	4.5	4.3	4.4
8	4.7	4.8	4.75	31	4.9	4.2	4.55
9	4.5	4.	4.3	32	5.0	4.9	4.95
10	4.8	4.7	4.75	33	5.0	5.0	5.0
11	4.9	4.	4.7	34	4.25	5.25	4.75
12	5.0	4.8	4.9	35	4.2	5.2	4.7
13	4.8	4.8	4.8	36	4.8	4.8	4.8
14	5.2	5.3	5.25	37	5.0	5.5	5.25
15	4.9	5.0	4.95	38	5.0	4.9	4.95
16	4.8	5.0	4.9	39	4.8	4.8	4.8
17	4.9	4.7	4.8	40	4.8	4.9	4.85
18	5.0	4.8	4.9	41	5.0	4.8	4.9
19	5.0	4.8	4.9	42	4.8	4.5	4.65
20	5.2	5.3	5.1	43	5.0	4.5	4.75
21	4.9	4.9	4.9	44	5.0	4.8	4.9
22	5.0	4.2	4.6	44	5.0	4.8	4.9

A and B - two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM CRPV 2 (cutaneous fibropapilloma - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.7	4.9	4.8	23	5.0	4.6	4.8
2	4.7	4.8	4.75	24	4.6	5.0	4.8
3	4.9	5.0	4.95	25	4.5	4.9	4.7
4	4.7	5.1	4.9	26	4.7	4.9	4.8
5	4.5	4.6	4.55	27	4.7	4.0	4.35
6	4.0	4.5	4.25	28	4.0	5.5	4.75
7	5.0	4.5	4.75	29	4.0	4.9	4.45
8	4.9	4.5	4.7	30	4.9	4.0	4.45
9	4.9	4.5	4.7	31	4.0	5.0	4.5
10	4.8	4.6	4.7	32	4.3	4.9	4.6
11	4.7	5.2	4.95	33	4.5	4.5	4.4
12	5.0	5.2	5.1	34	4.2	4.6	4.4
13	4.9	5.0	4.95	35	4.2	5.0	4.6
14	4.7	4.8	4.7	36	4.7	5.3	5.0
15	4.9	5.1	5.0	37	4.3	5.2	4.8
16	5.0	5.2	5.15	38	5.2	4.3	4.75
17	4.8	4.9	4.85	39	4.5	5.0	4.75
18	4.7	4.9	4.8	40	5.0	4.3	4.65
19	4.6	5.1	4.85	41	4.8	4.9	4.85
20	5.0	5.0	5.0	42	5.1	5.2	5.15
21	5.0	4.9	4.95	43	5.1	4.9	5.0
22	4.9	5.9	5.4	44	4.5	4.9	4.7

A and B - two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of CRPV particle diameters.

EXTRACT FROM CRPV 2 (cutaneous fibropapilloma - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.5	4.9	4.7	33	4.3	5.2	4.75
2	4.5	4.0	4.75	34	4.7	4.5	4.6
3	4.9	4.5	4.7	35	4.8	5.0	4.9
4	4.7	4.7	4.85	36	5.2	4.7	4.95
5	5.5	4.7	4.9	37	5.0	5.2	5.1
6	4.8	5.2	5.0	38	5.2	4.8	5.0
7	4.8	5.0	4.9	39	4.8	4.9	4.85
8	4.5	4.9	4.7	40	4.8	4.9	4.85
9	5.2	4.9	4.9	41	5.0	5.3	5.15
10	4.3	4.9	4.6	42	5.1	4.9	5.0
11	4.7	5.0	4.85	43	5.2	4.6	4.9
12	4.5	5.0	4.75	44	5.0	4.5	4.75
13	4.8	4.7	4.7	45	4.5	5.0	4.75
14	4.9	4.6	4.75	46	4.8	5.2	5.0
15	4.9	4.4	4.65	47	4.7	5.3	5.0
16	5.0	4.8	4.9	48	4.7	4.2	4.45
17	5.2	5.2	5.1	49	4.0	4.5	4.25
18	4.5	5.0	4.75	40	5.0	4.8	4.9
19	5.0	4.4	4.7	41	4.9	4.0	4.45
20	5.0	5.0	5.0	42	4.5	4.6	4.55
21	5.2	4.6	4.9	43	4.0	3.9	3.95
22	4.0	5.1	4.6	44	3.9	4.2	4.05

A and B - two diameter measurements made at 90^{o} to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM CBPV 2 (cutaneous fibropapilloma - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	4.5	4.[illegible]	4.5				
2	4.0	4.[illegible]	4.1				
3	3.9	4.1	4.0				
4	4.[illegible]	4.1	4.15				
5	4.0	4.1	4.05				
6	3.8	4.0	3.9				
7	4.[illegible]	4.0	4.1				
8	3.9	4.[illegible]	4.05				
9	4.[illegible]	4.1	4.15				
10	4.[illegible]	4.0	4.1				
11	4.[illegible]	4.1	4.15				
12	4.0	4.[illegible]	4.15				
13	4.	4.	4.[illegible]				
14	4.1	4.0	4.0				
15	3.9	4.[illegible]	4.15				
16	4.1	4.[illegible]	4.[illegible]				
17	4.[illegible]	4.	4.1				
18	3.8	4.[illegible]	4.15				

A and B two diameter measurements made at 90° to each other.

APPENDIX B - Measurement of BPV particle diameters.

EXTRACT FROM DP (dog skin papilloma - single case)

Particle Number	Measurement (mm) A	B	Mean	Particle Number	Measurement (mm) A	B	Mean
1	5.0	[illegible]	[illegible]	23	5.5	5.9	5.7
2	4.9	[illegible]	[illegible]	24	5.6	5.5	5.55
3	5.0	[illegible]	[illegible]	25	5.5	5.3	5.4
4	[illegible]	[illegible]	[illegible]	26	6.0	5.0	5.5
5	[illegible]	[illegible]	[illegible]	27	[illegible]	6.2	5.7
6	[illegible]	[illegible]	[illegible]	28	5.6	5.8	5.7
7	[illegible]	[illegible]	[illegible]	29	5.6	6.0	5.8
8	[illegible]	[illegible]	[illegible]	30	6.0	5.2	5.6
9	[illegible]	[illegible]	[illegible]	31	5.7	6.1	5.9
10	[illegible]	[illegible]	[illegible]	32	6.1	5.2	5.65
11	[illegible]	[illegible]	[illegible]	33	5.5	6.0	5.75
12	[illegible]	[illegible]	[illegible]	34	6.2	6.5	5.95
13	[illegible]	[illegible]	[illegible]	35	5.3	6.0	5.65
14	[illegible]	[illegible]	[illegible]	36	6.0	4.8	5.4
15	[illegible]	[illegible]	[illegible]	37	5.0	5.5	5.25
16	[illegible]	[illegible]	[illegible]	38	5.9	5.8	5.85
17	[illegible]	[illegible]	[illegible]	39	5.6	5.8	5.7
18	[illegible]	[illegible]	[illegible]	40	5.8	5.8	5.8
19	[illegible]	[illegible]	[illegible]	41	5.8	5.5	5.65
20	[illegible]	[illegible]	[illegible]	42	5.2	4.9	5.05
21	[illegible]	[illegible]	[illegible]	43	5.0	6.0	5.5
22	[illegible]	[illegible]	[illegible]	44	5.0	6.2	5.6
				45	5.6	5.3	5.45
				46	5.8	5.6	5.7
				47	5.2	5.9	5.55
				48	5.6	6.0	5.8
				49	5.0	5.8	5.4
				50	5.8	5.9	5.85

A and B two diameter measurements made at 90° to each other.

APPENDIX B Measurement of BPV particle diameters

Calculation of true magnification

Standard grid has		2160 lines/mm
therefore actual known distance between lines		0.000463mm
electronmicrograph mean measured distance		3.969cm
	SD	± 0.0612cm
	number	75

$$\text{true magnification} = \frac{39.7}{0.000463}$$

$$= 85,760 \text{ X}$$

Conversion of BPV particle measurements

Actual, on electronmicrograph measurements were divided by the calculated true magnification, 85, 760 to give actual BPV particle diameters in mμ. as tabulated in table 21 in thesis text.

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 68882

Week post infection	Red Blood Cells	White Blood Cells	Haemoglobin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^9$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$/ml			
0	5.99	5,600	10.0	35	26	0	74	0	1416	0	4144	0
2	5.39	5,300	9.4	32	43	2	48	7	2279	106	2544	371
4	4.76	6,600	8.2	27	49	0	51	0	3234	0	3366	0
5	5.74	5,800	10.4	34								
6	5.47	7,000	10.0	31	38	3	58	1	2660	210	4060	70
8	4.15	5,600	10.7	33	45	0	55	0	2520	0	3080	0
10	6.01	5,300	11.0	33	17	0	82	1	901	0	4346	53
11	5.30	7,000	10.7	31								
12	6.04	6,200	10.4	32	33	2	65	0	2046	124	4030	0
14	4.65	3,900	7.2	26	21	2	75	2	819	78	2925	78
16	4.84	6,800	7.9	30	32	2	66	0	2176	136	4488	0
18	5.65	7,800	7.5	32	31	2	67	0	2418	156	5226	0
20	5.64	8,600	7.5	30								
23	5.30	6,100	9.1	32	34	3	63	0	2074	183	3843	0
24	5.49	8,000	9.1	34	38	0	62	0	3040	0	4960	0

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 68886

Week post infection	Red Blood Cells	White Blood Cells	Haema-globin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^9$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$/ml			
0	5.27	4,600	7.2	24	13	0	87	0	598	0	4002	0
2	7.24	8,300	11.6	34	42	0	55	3	3486	0	4565	249
4	5.64	6,500	7.5	24	46	2	52	0	2990	130	3380	0
5	7.23	7,300	9.5	30								
6	6.10	8,700	9.7	30	52	2	46	0	4524	174	4002	0
8	6.74	6,700	9.8	30	34	2	62	2	2278	134	4154	134
10	6.02	7,300	8.8	29	22	1	77	0	1606	73	5621	0
11	6.57	9,700	8.5	30								
12	6.64	7,300	10.0	28	40	0	58	2	2920	0	4234	146
14	7.78	6,000	7.9	30	40	1	57	2	2400	60	3420	120
16	6.76	7,200	8.5	30	32	0	65	3	2304	0	4680	216
18	6.88	8,100	7.9	32	16	3	81	0	1296	243	6561	0
20	7.19	9,500	7.9	32								
23	6.57	9,800	8.5	30	36	2	62	0	3528	196	6076	0
24	9.07	7,200	11.0	42	36	1	62	1	2592	72	4464	72

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 68888

Week post infection	Red Blood Cells	White Blood Cells	Haema-globin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$x 10^9$/ml	$x 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $x 10^6$/ml			
0	6.42	6,000	7.5	27	38	2	60	0	2228	120	3600	0
2	7.90	7,900	10.4	32	38	1	60	1	3002	79	4740	79
4	7.24	11,900	9.1	29	52	2	46	0	6188	238	5474	0
5	7.90	11,600	10.0	33								
6	6.88	8,600	10.4	32	23	0	74	1	1978	0	6364	86
8	4.65	7,000	10.4	29	34	0	62	4	2380	0	4340	280
10	7.05	6,800	9.7	31	31	2	67	0	2108	136	4556	0
11	7.97	9,000	10.0	32								
12	7.60	13,600	10.4	32	34	0	66	0	4624	0	8976	0
14	5.66	5,500	7.9	28	54	2	44	0	2970	110	2420	0
16	7.73	10,900	7.5	31	42	0	56	2	4578	0	6104	218
18	8.04	12,700	8.2	31	37	0	62	1	4699	0	7874	127
20	8.70	10,300	7.0	31								
23	8.41	9,700	8.8	30	26	2	72	0	2522	194	6984	0
24	10.46	7,300	10.0	40	31	1	66	2	2263	73	4818	146

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 66893

Week post infection	Red Blood Cells	White Blood Cells	Haemoglobin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$x 10^9$/ml	$x 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $x 10^6$/ml			
0	7.33	6,100	7.9	25	40	1	59	0	2440	61	3599	0
2	8.11	10,900	10.0	32	42	3	52	3	4578	327	5668	327
4	6.69	8,500	8.5	25	40	1	59	0	3400	85	5015	0
5	11.81	12,100	10.0	30								
6	6.63	8,700	10.0	29	45	0	55	0	3915	0	4785	0
8	7.79	9,500	11.6	36	48	1	51	0	4560	95	4845	0
10	6.50	9,700	10.4	30	19	1	80	0	1843	97	7760	0
11	7.24	9,200	10.7	32								
12	7.36	13,000	10.4	31	33	0	66	1	4290	0	8580	130
14	5.75	9,700	7.9	28	47	2	47	4	4559	194	4559	388
16	6.99	11,100	7.9	27	35	0	65	0	3885	0	7215	0
18	7.20	11,900	8.5	33	21	2	74	3	2499	238	8806	357
20	7.97	12,600	7.0	30								
23	7.38	14,600	8.8	29	18	0	79	3	2628	0	11534	438
24	7.32	8,600	9.4	35	10	2	88	0	860	172	7568	0

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 68891

Week post infection	Red Blood Cells	White Blood Cells	Haemaglobin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^6$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$/ml			
5	8.25	14,300	8.5	29	35	1	64	0	5005	143	9152	0
7	8.38	11,700	8.8	29	26	2	72	0	3042	234	8424	0
9	9.00	10,600	7.8	28	29	1	70	0	3074	106	7420	0
12	7.71	10,500	9.4	30	48	2	47	3	5040	210	4935	315
14	6.12	12,600	8.8	27	57	0	43	0	7182	0	5418	0
15		10,300	11.0	30								
16	8.17	9,700	11.0	34	40	2	57	1	3880	194	5529	97
18	7.63	8,800	11.4	32	41	0	59	0	3608	0	5192	0
34	7.73	6,500	9.1	32	54	0	46	0	3510	0	2990	0

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 68745

Week post infection	Red Blood Cells	White Blood Cells	Haemaglobin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^9$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$/ml			
5	8.42	8,400	9.1	30	17	0	82	1	1428	0	6888	84
7	9.18	10,400	9.7	31	25	1	73	1	2600	104	7592	104
9	7.80	10,500	7.5	28	33	0	67	0	3465	0	7035	0
12	8.88	6,800	11.6	35	38	0	56	6	2584	0	3808	408
14	7.63	10,600	9.7	29	55	0	45	0	5830	0	4770	0
15	7.67	10,700	10.7	31								
16	8.66	7,600	10.0	30	31	0	66	3	2356	0	5016	228
18	8.27	8,300	10.7	33	35	1	66	0	2905	83	5478	0
34	9.27	12,300	11.0	39	49	2	49	0	6027	246	6027	0

HAEMATOLOCICAL RESULTS FOR CALF NUMBER 81

Week post infection	Red Blood Cells	White Blood Cells	Haemo-globin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^9$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$ ml			
0	8.15	4,000	9.4	35	38	1	58	3	1520	40	2320	120
2	6.92	8,000	9.1	31	50	0	47	2	4000	0	3760	160
3	7.32	8,000	9.8	31								
4	7.35	6,500	9.7	32	36	0	63	1	2340	0	2268	65
6	8.21	4,200	9.2	19	38	1	57	4	1596	42	2394	168
8	6.45	6,000	8.5	27	33	2	65	0	1980	120	3900	0
9	6.42	5,200	6.6	27								
10	7.52	5,400	10.0	31	37	0	61	2	1998	0	3294	108
12	6.77	7,500	5.7	24	41	1	58	0	3075	75	4350	0
14	6.02	6,800	7.2	26	46	1	53	0	3128	68	3604	0
16	7.51	7,400	7.9	33	39	0	60	1	2886	0	4440	74
18	7.31	8,800	9.8	31								
21	6.89	7,800	8.2	30	31	0	69	0	2418	0	5382	0

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 82

Week post infection	Red Blood Cells	White Blood Cells	Haema-globin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^9$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$/ml			
0	9.41	6,000	11.3	40	43	4	51	2	2580	240	3060	120
2	9.63	11,400	11.6	42	50	0	46	4	5700	0	5244	456
3	9.70	8,400	12.0	40								
4	9.66	7,500	11.3	37	31	0	66	3	2325	0	4950	225
6	8.66	5,400	10.7	23	30	0	68	2	1620	0	3672	108
8	7.79	6,200	10.4	34	34	1	65	0	2108	62	4030	0
9	7.72	6,700	9.1	31								
10	9.18	6,300	12.5	35	28	1	71	0	1764	63	4473	0
12	7.79	7,700	8.8	30	39	2	58	1	3003	154	4466	77
14	7.58	9,000	7.5	29	44	0	56	0	3960	0	5040	0
16	6.71	8,500	7.5	30	28	1	70	1	2380	85	5950	85
18	CLOTTED											
21	7.18	8,300	8.2	31	25	1	74	0	2075	83	6142	0

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 83

Week post infection	Red Blood Cells	White Blood Cells	Haema-globin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^9$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$/ml			
0	6.42	5,500	10.0	34	37	1	60	2	2035	55	2220	110
2	6.34	9,700	9.4	30	58	0	41	1	5626	0	3977	97
3	5.93	10,500	9.0	26								
4	6.46	8,900	10.0	31	25	0	75	0	2225	0	6675	0
6	5.90	6,100	8.8	26	22	2	72	4	1342	122	4392	244
8	5.84	8,200	9.4	28	29	9	62	0	2378	738	5084	0
9	5.74	7,700	8.2	28								
10	6.33	8,700	10.4	30	30	1	69	0	2610	87	6003	0
12	5.15	11,000	7.5	25	29	2	69	0	3190	220	7590	0
14	5.01	7,500	6.6	25	21	0	79	0	1575	0	5925	0
16	6.12	9,000	7.9	32	31	1	68	0	2790	90	6120	0
18	6.19	10,300	8.5	30								
21	5.65	8,200	7.5	27	32	0	68	0	2624	0	5576	0

HAEMATOLOGICAL RESULTS FOR CALF NUMBER 84

Week post infection	Red Blood Cells	White Blood Cells	Haemoglobin	Packed Cell Volume	% Neutrophils	% Eosinophils	% Lymphocytes	% Monocytes	Number Neutrophils	Number Eosinophils	Number Lymphocytes	Number Monocytes
	$\times 10^9$/ml	$\times 10^6$/ml	gm/100ml	ml/100ml	Differential Cell Count (Relative)				Differential Count (Absolute) $\times 10^6$/ml			
0	9.48	7,800	10.7	36	41	0	54	5	3198	0	4212	390
2	6.68	11,200	8.8	28	67	3	30	0	7504	336	3360	0
3	7.67	7,700	9.5	30								
4	7.44	6,700	9.1	29	46	0	53	0	3082	0	3551	0
6	6.67	4,300	7.9	24	30	1	68	1	1290	43	2924	43
8	5.87	5,200	9.4	25	36	5	59	0	1872	260	3068	0
9	6.44	5,000	7.9	24								
10	6.79	5,200	8.8	28	31	2	67	0	1612	104	3484	0
12	5.97	6,400	7.5	25	40	2	58	0	2560	128	3712	0
14	6.38	6,600	6.6	24	42	0	58	0	2772	0	3828	0
16	6.50	6,900	6.6	27	32	1	67	0	2208	69	4623	0
18	6.78	8,100	8.8	26								
21	6.09	6,700	6.9	24	28	0	71	1	1876	0	4757	67

Appendix D: Detailed results of multiple intradermal injections of various dilutions of pasteurised and untreated BPV isolates

MAJOR SKIN SITE	VIRUS DILUTION	VIRUS ON SITE AREA	CALF NUMBER AND ISOLATE USED									
			68882		68886		68888		68891		68893	
VIRUS TYPE			RG		BPV2		F		BPV1		FR	
Mean particle diameter ± S.D.			47.6± 2.9		54.9 ± 3.6		57.6± 2.6		53.6± 4.5		53.0± 4.1	
in vivo measure*			A	B	A	B	A	B	A	B	A	B
Left Neck	10^{-1}	Pasteurised	$^{7}/_{7}$	$^{0}/_{10}$	$^{7}/_{7}$	$^{10}/_{10}$	$^{7}/_{7}$	$^{0}/_{10}$	$^{7}/_{7}$	$^{7}/_{7}$	$^{7}/_{7}$	$^{7}/_{7}$
		Untreated	$^{7}/_{7}$	$^{0}/_{10}$	$^{7}/_{7}$	$^{10}/_{10}$	$^{7}/_{7}$	$^{0}/_{10}$	$^{7}/_{7}$	$^{7}/_{7}$	$^{7}/_{7}$	$^{7}/_{7}$
Left Shoulder	10^{-2}	Pasteurised	$^{6}/_{10}$	$^{0}/_{10}$	$^{8}/_{10}$	$^{10}/_{10}$	$^{6}/_{10}$	$^{0}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{5}/_{10}$	$^{7}/_{10}$
		Untreated	$^{6}/_{10}$	$^{0}/_{10}$	$^{7}/_{10}$	$^{10}/_{10}$	$^{8}/_{10}$	$^{0}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{4}/_{10}$	$^{6}/_{10}$
Left Rump	10^{-3}	Pasteurised	$^{2}/_{10}$	$^{0}/_{10}$	$^{1}/_{10}$	$^{4}/_{10}$	$^{2}/_{10}$	$^{0}/_{10}$	$^{9}/_{10}$	$^{10}/_{10}$	$^{2}/_{10}$	$^{3}/_{10}$
		Untreated	$^{3}/_{10}$	$^{0}/_{10}$	$^{3}/_{10}$	$^{3}/_{10}$	$^{1}/_{10}$	$^{0}/_{10}$	$^{8}/_{10}$	$^{8}/_{10}$	$^{2}/_{10}$	$^{3}/_{10}$

* A - number of injection sites with papillomas/number of injection sites
B - number of injection sites with fibromas/number of injection sites
\+ - calculated by the method of Reed and Muench (5)
n.a. - not applicable.

Appendix D: Detailed results of multiple intradermal injections of various dilutions of pasteurised and untreated BPV isolates (Cont'd.)

MAJOR SKIN SITE	VIRUS DILUTION	VIRUS ON SITE AREA	CALF NUMBER AND ISOLATE USED									
			68882		68886		68888		68891		68893	
VIRUS TYPE			RG		BPV2		F		BPV1		FR	
Mean particle diameter ± S.D.			47.6± 2.9		51.7± 4.8		57.6± 2.6		53.6± 4.5		53.0± 4.1	
in vivo measure*			A	B	A	B	A	B	A	B	A	B
Right Rump	10^{-4}	Pasteurised	0/10	0/10	0/10	2/10	0/10	0/10	3/10	4/10	0/10	0/10
		Untreated	0/10	0/10	0/10	1/10	0/10	0/10	2/10	5/10	0/10	0/10
Right Shoulder	10^{-5}	Pasteurised	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		Untreated	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Right Neck	10^{-6}	Pasteurised	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		Untreated	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
+		Pasteurised	2.3	n.a.	2.4	3.1	2.3	n.a.	3.8	3.9	2.2	2.5
in vivo titre		Untreated	2.4	n.a.	2.5	2.9	2.4	n.a.	3.5	3.8	2.1	2.4

* A - number of injection sites with papillomas/number of injection sites
B - number of injection sites with fibromas/number of injection sites
\+ - calculated by the method of Reed and Muench (5)
n.a. - not applicable.

APPENDIX E BOVINE PAPILLOMA VIRUS IN VITRO TRANSFORMATION ASSAY RESULTS.

ASSAY NUMBER	SAMPLE	CELLS	NUMBER OF REPLICATES	LOG_{10} TD50
1	65819 (1)	11S	4	3.16
2	65819 (2)	11S	4	3.24
3	65819 (3)	11S	4	3.16
4	65819 (4)	11S	4	3.54
5	65819 (5)	11S	4	3.24
6	65819 (6)	11S	4	3.31
7	65819 (7)	11S	4	3.54
8	65819 (8)	11S	4	4.24
9	65819 (9)	11S	4	3.45
10	65819 (10)	11S	4	3.53
11	65819 (1)	12S	4	3.47
12	65819 (2)	12S	4	3.55
13	65819 (3)	12S	4	3.60
14	65819 (4)	12S	4	3.68
15	65819 (5)	12S	4	3.55
16	65819 (6)	12S	4	3.47
17	65819 (7)	12S	4	3.78
18	65819 (8)	12S	4	3.55
19	65819 (9)	12S	4	3.68
20	65819 (10)	12S	4	3.47
21	65819 (1)	12C	4	4.02
22	65819 (2)	12C	4	3.60
23	65819 (3)	12C	4	3.47
24	65819 (4)	12C	4	3.55
25	65819 (5)	12C	4	3.60
26	65819 (6)	12C	4	3.83
27	65819 (7)	12C	4	3.60
28	65819 (8)	12C	4	3.47
29	65819 (9)	12C	4	3.55
30	65819 (10)	12C	4	3.55

ASSAY NUMBER	SAMPLE	CELLS	NUMBER OF REPLICATES	LOG_{10} TD50
31	66702 (1)	12S	4	4.15
32	66702 (2)	12S	4	4.21
33	66702 (3)	12S	4	4.15
34	66702 (4)	12S	4	4.30
35	66702 (5)	12S	4	4.23
36	66702 (6)	12S	4	4.11
37	66702 (7)	12S	4	4.27
38	66702 (8)	12S	4	4.17
39	66702 (9)	12S	4	4.33
40	66702 (10)	12S	4	4.30
41	66702 (1)	11S	4	4.30
42	66702 (2)	11S	4	4.11
43	66702 (3)	11S	4	4.17
44	66702 (4)	11S	4	4.23
45	66702 (5)	11S	4	4.17
46	66702 (6)	11S	4	4.23
47	66702 (7)	11S	4	4.30
48	66702 (8)	11S	4	4.15
49	66702 (9)	11S	4	4.38
50	66702 (10)	11S	4	4.33
51	66702 (1)	12C	4	4.23
52	66702 (2)	12C	4	4.17
53	66702 (3)	12C	4	4.30
54	66702 (4)	12C	4	4.11
55	66702 (5)	12C	4	4.30
56	66702 (6)	12C	4	4.27
57	66702 (7)	12C	4	4.17
58	66702 (8)	12C	4	4.11
59	66702 (9)	12C	4	4.17
60	66702 (10)	12C	4	4.23

ASSAY NUMBER	SAMPLE	CELLS	NUMBER OF REPLICATES	LOG_{10} TD50
61	66702 (1)	11S	5	4.1
62	66702 (1)	11S	5	4.1
63	66702 (1)	11S	5	3.9
64	66702 (1)	11S	5	4.1
65	66702 (1)	11S	5	4.1
66	65819 (1)	13M	5	3.3
67	65819 (2)	13M	5	3.7
68	65819 (3)	13M	5	3.1
69	66702 (1)	13M	5	4.3
70	53833	2M	16	4.5
71	53833	5M	12	4.3
72	66702 F	5M	4	6.0
73	66702 PF	5M	5	4.2
74	66702 PF	11S	5	4.1
75	Dog Papilloma	11S	10	0
76	"	12C	10	0
77	"	12S	10	0
78	"	2M	10	0
79	RGA	11S	10	0
80		12C	10	0
81		12S	10	0
82		2M	10	0
83		13M	10	0
84	RGB	11S	10	0
85		12C	10	0
86		12S	10	0
87		2M	10	0
88		13M	10	0
89	FR	11S	10	3.1
90		12C	5	2.7

ASSAY NUMBER	SAMPLE	CELLS	NUMBER OF REPLICATES	LOG_{10} TD50
91		12S	5	2.7
92		2M	5	3.1
93		13M	5	2.9
94	FA	11S	10	0
95	FB	11S	10	0
96	FA	12S	10	0
97		12C	10	0
98	FB	12S	10	0
99		12C	10	0
100	APV(20% CS)	11S	5	1.5
101	APV(SS)	11S	5	2.7
102	APV(Post 10)	11S	5	1.5
103	FA 11SI	11S	5	3.1
104	Hamster F.	11S	5	0
105	T 13	11S	5	0
106	66702 purified	11S	5	5.8
107	DP purified	11S	5	0
108	RPCPV	11S	5	2.9
109	RGA purified	11S	5	0
110	66702 n.p.	11S	5	4.1
111	FC	11S	5	0
112	RGC	11S	5	3.1
113	FRC	11S	5	2.7
114	RGA purified	11S	5	0
115	RGA crude	11S	5	0
116	FR purified	11S	5	4.1
117	66702 n.v.	12C	5	4.75
118	68891 Men	12C	5	0
119	82 Men	12C	5	0
120	68888 W16	12C	5	0

ASSAY NUMBER	SAMPLE	CELLS	NUMBER OF REPLICATES	LOG_{10} TD50
121	68893 W16	12C	5	2.9
122	68882 W16	12C	5	0
123	68891 W16	12C	5	3.7
124	NMT	12C	5	0
125	NMT	12S	5	0
126	NMT	11M	5	0
127	NMT	13M	5	0
128	68886 W16	12C	5	4.5